University of Alberta

Studies on fungal disease resistance in *Brassica*

by

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ABSTRACT

Plant diseases caused by fungi are considered to be important factors that severely limit food production. *Brassica* species such as canola, one of the important agricultural crops, are affected by several abiotic and biotic stresses. Phoma stem canker (also known as blackleg) and Alternaria blackspot of canola caused by *Leptosphaeria maculans* and *Alternaria brassicae*, respectively are important diseases of canola. In the case of blackleg, resistant varieties of canola were developed but resistance breakdown has been observed due to the evolution of pathogenic isolates of *L. maculans*. Resistance to blackspot disease has not been identified in *Brassica* species. Therefore, elucidating the details of fungal-plant pathogenesis, including the molecular mechanisms of these diseases and the nature of the host cellular response is important. In recent years, proteomic analysis has emerged as a powerful approach to study, not only defense-related proteins that are induced in pathogen-challenged plants to overcome disease, but also proteins that are produced by phytopathogens and serve as virulence or pathogenicity factors. We performed a proteome-level investigation with different disease-susceptible and -tolerant *Brassica* species using two-dimensional electrophoresis (2-DE) and mass spectrometry (MS). Several proteins identified in these studies were those with antioxidant activity, photosynthesis-related and other metabolic enzymes. The expression of genes encoding some of these proteins was studied using quantitative real-time PCR (q-RT-PCR) and Western blotting. Our findings suggested that disease-tolerant plants appear to activate a large group of antioxidant enzymes and pathways involved in the reinforcement of plant cell

walls. Proteome-level studies were also conducted to understand the possible basis for differential virulence between two isolates of *A. brassicae* varying in their virulence. The distinct protein profiles obtained from these studies may help to reveal the nature of the molecular determinants of virulence that could be further exploited to identify pathogenicity factors and novel targets for managing disease. We also investigated the effects of cytokinins on the two pathogens. Our *in planta* results suggest the delayed onset of both diseases by the exogenous application of cytokinin. Our results may have important bearing on the development of novel crop protection strategies.

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LIST OF ABBREVIATIONS

2-DE	**Two dimensional electrophoresis**
ABA	**Abscisic acid**
ABC	**ATP-binding cassette**
ANOVA	**Analysis of variance**
APX	**Ascorbate peroxidase**
ATP	**Adenine diphosphate**
BAP	**6-Benzyl amino purine**
CA	**Carbonic anhydrase**
CAT	**Catalase**
CAD	**Cinnamyl alcohol dehydrogenase**
CBB	**Colloidal brilliant blue**
CDD	**Conserved domain database**
CHAPS	**3-[(3-cholamidopropyl) dimethyl-ammonio]1-propane sulphonate**
CHCA	**alpha-cyano-4-hydoxycinnamic acid**
CK	**Cytokinin**
CYP	**Cyclophillin**
DIGE	**Difference in-gel electrophoresis**
DMSO	**Dimethyl sulfoxide**
DNA	**Deoxyribonucleic acid**
dNTP	**Deoxyribonucleotide triphosphate**
DPX	**di-n-butylphtalate in xylene**

DTT	**Dithiothreitol**
EDTA	**Ethylene diamine tetraacetic acid**
ELISA	**Enzyme-linked immunosorbent assay**
ER	**Expression ratio**
ESI	**Electrospray ionization**
EST	**Expressed sequence tag**
FAA	**Formalin aceto alcohol**
FAD	**Flavin adenine dinucleotide**
FMN	**Flavin mononucleotide**
FTICR	**Fourier Transform Ion Cyclotron Resonance**
GAPDH	**Glyceraldehyde-3-phosphate dehydrogenase**
GFP	**Green fluorescent protein**
GLM	**Generalized linear model**
GLP	**Germin-like protein**
GRP	**Glycine rich protein**
GSH-Px	**Glutathione peroxidase**
HCL	**Hydrochloric acid**
HPLC	**High performance liquid chromatography**
HR	**Hypersensitive reaction**
IAA	**Indole-3-acetic acid**
IAA	**Iodoacetamide**
ICAT	**Isotope-coded affinity tag**
IEF	**Isoelectric focusing**

IP	**Interaction phenotype**
IPG	**Immobilized pH gradient**
IPM	**Integrated pest management**
ISR	**Induced systemic resistance**
iTRAQ	**Isobaric tagging for relative and absolute quantitation**
JA	**Jasmonic acid**
KCL	**Potassium chloride**
LC	**Liquid chromatography**
LC-MS	**Liquid chromatography-mass spectrometry**
m/z	**Mass-to-charge ratio**
MALDI	**Matrix-assisted laser desorption ionization**
MAPK	**Mitogen-activated protein kinases**
MDH	**Malate dehydrogenase**
MS	**Mass spectrometry**
MudPIT	**Multidimensional protein identification technology**
MW	**Molecular weight**
NAA	**α-naphthaleneacetic acid**
NaGLP	***Nicotiana attenuata* germin-like protein**
NaOH	**Sodium hydroxide**
NDPK	**Nucleoside diphosphate kinase**
NRPS	**Nonribosomal peptide synthetase**
PAGE	**Polyacrylamide gel electrophoresis**
PCD	**Programmed cell death**

PCR	**Polymerase chain reaction**
PDA	**Piperazine diacrylamide**
PG	**Pathogenicity group**
PGK	**Phosphoglycerate kinase**
PPIase	**Peptidylprolyl isomerase**
PR	**Pathogenesis-related**
Prxs	**Peroxiredoxins**
PTM	**Post-translational modifications**
PVDF	**Polyvinylidene difluoride**
q-RT-PCR	**Quantitative real time -PCR**
QTL	**Quantitative trait loci**
RFLP	**Restriction fragment length polymorphism**
RH	**Relative humidity**
RNA	**Ribonucleic acid**
ROS	**Reactive oxygen species**
SA	**Salicylic acid**
SAR	**Systemic acquired resistance**
SAS	**Statistical analysis system**
SDS	**Sodium dodecyl sulfate**
SOD	**Superoxide dismutase**
TBP	**Tributylphosphine**
TCA	**Trichloroacetic acid**
TEMED	**N, N, N', N'-tetramethylethylenediamine**

TFA	**Trifluoroacetic Acid**
TMB	**3, 3', 5, 5'-tetramethylbenzidine**
TNV	**Tobacco necrosis virus**
TOF	**Time of flight**
UV	**Ultraviolet**
VIGS	**Virus-induced gene-silencing**
ZR	**Zeatin riboside**

1. Review of literature

1.1. General introduction

Agriculture, including plant-based food production, is vital in order to sustain the needs of an ever-growing population. According to estimates published by the United States Census Bureau, the earth's population has already hit the 6.5 billion mark in February, 2006 (http://www.census.gov) and is expected to increase to 8.3 billion by 2030 at an average growth of 1.1 percent per year till 2030 (source: Food and Agriculture Organization; www.fao.org). There will be less expansion of farmland for food production especially in developing countries where most of the suitable land is already in use and hence, cropland expansion will result in using forest land (source: Food and Agriculture Organization; www.fao.org). However, plants are constantly challenged by stresses, imposed by unfavorable environmental conditions, insects, weeds and pathogens (such as fungi, viruses, bacteria, nematodes etc.). Plant diseases are very important owing to the constraints they place on plant productivity. In the past, diseases have resulted in massive crop losses, such as late blight of potato caused the great Irish famine in 1845-1846; ergot of rye and wheat; powdery mildew of grapes in 1840-1850s; sugarcane mosaic caused by virus, bacterial soft-rot of vegetables led to the huge losses of fleshy vegetables and nematodal root knot disease caused severe losses worldwide (Agrios, 1998). The understanding of plant-pathogen interactions and the improvement of the ability of plants to tolerate pathogens are crucial to avoid a repetition of history.

Advances in plant breeding approaches resulted in the development of high-yielding crop varieties. However, the continual urge to develop higher yielding crop varieties, coupled with the immediate need to control the heavy damages inflicted by pests and pathogens resulted in an over-reliance on chemical fertilizers and pesticides. The evolution of resistant strains of pathogens and the realization of widespread ecological damage caused by the strategies based on chemical intervention soon forced both plant pathologists and entomologists to emphasize non-pesticide based control strategies. These approaches included genetic host resistance to pathogens and pests, cultural control practices such as tillage and rotation, and pathogen-free seed or planting stock (Jacobsen, 1997). Present agricultural systems are therefore based on the paradigm of Integrated Pest Management (IPM), defined as "a sustainable approach to manage pests (and pathogens) by combining biological, cultural, physical, and chemical tools in a way that minimizes economic, health and environmental risks" (Jacobsen, 1997). Such programs strive to achieve a level of disease control that is acceptable in economic terms and simultaneously causes minimal disturbance to non-target individuals in the crop's ecosystem (Fry, 1982). Thus, IPM not only includes the understanding of the basic principles of plant pathology, but it also combines this with healthy farm practices such as providing a balanced crop nutrition, practicing appropriate farm sanitation principles, managing crop residues to minimize pathogen carryover into subsequent crops and developing a sound crop rotation strategy as well as the use of resistant varieties. One of the best documented success stories was exemplified by the Campbell Soup Company that initiated an IPM-based program in collaboration with tomato and celery growers in the U.S. and Mexico in 1984. By

1994, it was reported that the pesticide use by the growers had been reduced by ca. 50 % without any loss of yield or quality (Bolkan and Reinert, 1996).

Plants have defense systems relying on combinations of constitutive and induced mechanisms (Hammond-Kosack and Jones, 1996; Lamb, 1994) to resist pathogen attack. These naturally occurring defense systems may be manipulated either via classical breeding or genetic engineering to improve the tolerance of plants to diseases. With genetic engineering, it is possible to introduce genes from different plant species (Broekaert *et al.*, 1995) or kingdoms (Rao, 1995) into crops. Despite this, an ongoing challenge for the crop protection industry is to ensure the development of the strategies that effectively provide broad-spectrum and durable resistance to plant diseases. Plant pathogens evolve, often at frequencies much higher than the host plant, constantly resulting in new strains which break down the host's ability to tolerate them. Understanding the molecular responses of plants during compatible and incompatible interactions may generate new information on the mechanisms underlying tolerance or susceptibility to a pathogen. Such information may prove to be crucial in the development of robust, disease-tolerant plants.

Recent applications of state-of-the-art molecular biology tools has resulted in the identification of several genes involved in mediating plant responses to pathogens. The rapid progress in the completion of whole genome sequencing projects and the availability of expressed sequence tag (EST) databases has opened new avenues for analyzing biological systems and their complex functions (Canovas *et al.*, 2004). Similarly, mass spectrometry-based proteomics has become a powerful and increasingly popular approach to not only identify proteins that are involved during

these plant-pathogen interactions, but also to better understand their biology (Martin *et al.*, 2003; Canovas *et al.*, 2004; Padliya and Cooper, 2006). The research presented in this dissertation demonstrates the utility of proteomics-based strategies to characterize the responses of canola (*Brassica napus* L.) to two pathogens that have the potential to cause devastating losses to this crop. We begin with a literature review and a statement of objectives, after which the individual studies and the results obtained are presented and discussed.

1.2. Significance of plant diseases

Generally, a plant is considered to be healthy or normal when it is able to carry out its physiological functions to the best of its genetic potential. However, it is difficult to pinpoint exactly when a plant is not able to perform its best and can be classified as diseased. Hence, disease in plants is generally defined as "the series of visible and invisible responses of plant cells and tissues to a pathogenic microorganism or environmental factor that results in adverse changes in form, function, or integrity of the plant and leads to partial impairment or death of the plant or its parts" (Agrios, 1998).

Plant diseases are categorized as infectious (biotic) and non-infectious (abiotic) diseases. Diseases caused by unfavorable environmental variables such as temperature, moisture, light, oxygen, pH, mineral toxicities and nutrient deficiencies are considered as abiotic (Agrios, 1998). On the other hand, fungi, prokaryotes (bacteria and mollicutes), viruses, viroids, nematodes, protozoa and parasitic higher

plants are the causal agents of biotic diseases, with the majority of plant diseases being caused by fungi (Montesinos, 2002).

Biotic diseases are destructive in nature because of their infectious nature and some of the major disease outbreaks among food crops have even led to famines and mass migrations. Perhaps, the most noteworthy example is the potato blight caused by *Phytophthora infestans* (1845-1849) that devastated Ireland by destroying 100 % of the Irish potato crop, contributing to the death of millions dependent on potato as the staple diet (Brouke, 1964). Losses from plant diseases have a significant economic impact, causing a reduction in income for crop producers and higher prices for consumers. Despite modern crop protection practices, it is estimated that around 10% of the world's harvest is still lost due to diseases (Strange and Scott, 2005). Therefore, strategies aimed at minimizing such losses are important towards achieving the goal of providing more affordable and adequate nutrition to every individual on Earth.

1.2.1. Host-pathogen interactions

Plant disease is the result of interactions between the plant host, the pathogen and the environment in which the disease develops and progresses. Some plants are completely resistant to certain pathogens as they employ different defense strategies for preventing disease from occurring and constitute an incompatible interaction with the pathogen. However, some pathogens can overcome the natural resistance mechanism of a particular host and the plant is considered to be susceptible to that pathogen. The pathogen itself is considered virulent and the interaction between the

virulent pathogen and susceptible host is considered to be a compatible interaction, which leads to disease. Plants and pathogens are often highly co-evolved. Pathogens focus on the colonization of the host and utilization of resources from the host, while plants are adapted to detect the presence of pathogens and to react with antimicrobial defenses and other stress responses which are discussed in the following sections (Wan *et al.*, 2002).

1.2.1.1. Weapons used by pathogens to attack plants

Pathogens attack plants using several strategies and have acquired the ability to survive on host tissue during their evolution. Therefore, for a disease to occur, pathogen exerts mechanical as well as chemical forces on host-tissues (Kosuge and Nester, 1984). Mechanical forces generated by appressorium (Dean, 1997) help in the penetration of the host tissue by the fungal pathogen where they secrete enzymes to degrade host's cell wall to make penetration process easier for the pathogen. While mechanical forces aid pathogen penetration into the host plant, metabolites (e.g. toxins, growth regulators and polysaccharides) synthesized by the pathogen in a constitutive or inducible manner interfere with the normal functions of the host plant (Goodman *et al.*, 1986). Enzymes involved in cell-wall degradation of a plant include cutinases, pectinases, cellulases, hemicellulases and ligninases (Collmer and Keen, 1986; Kolattukudy, 1985).

As indicated earlier, in addition to enzymes, pathogens also secrete several host- and non-host-specific toxins upon coming in contact with plants (Daly and Deverall, 1983). Examples of non-host-specific toxins are tentoxin, produced by the fungus *Altenaria alternata* (Meyer *et al.,* 1974), tabtoxin, by the bacterium *Pseudomonas syringae* pv *tabaci* (Braun, 1955), zinniol, by the fungus *Phoma macdonaldii*, cercosporin, by *Cercospora beticola*, fusicoccin, by *Fusicoccum amygdali*, and brefeldin A, by *Alternaria carthami*. Some of the host-specific toxins are victorin, produced by the fungus *Cochliobolus victoriae* (Walton and Panacione, 1993), peritoxin A and B, by *Periconia circinata* (Macko *et al.,* 1992), destruxin B, by *A. brassicae* (Bains and Tewari, 1987; Buchwaldt and Green, 1992) and phomalide, produced by *L. maculans* (Pedras and Biesenthal, 1998). Pathogens may also produce growth regulators such as auxins, cytokinins, gibberellins as well as inhibitors of growth regulators (Kuriger and Agrios, 1977; Stall and Hall, 1984; Yamada, 1993). In some cases, pathogens also release varying amounts of mucilaginous substances to facilitate pathogenesis by promoting colonization and enhancing survival of the pathogen inside the plant (Agrios, 1998). For example, in *M. grisea,* appresoria are tightly attached to the surfaces of coleoptile and leaf sheaths by mucilage (Koga, 1994 and 2001).

1.2.1.2. Plant defenses against pathogen

Plants defend themselves against pathogens by a combination of morphological adaptations and biochemical reactions (Bailey and Deverall, 1983).

Pre-existing structural and chemical defenses act as the first line of defense and include a waxy coating and a thick layer of hair (trichome) on the plant surface, both of which prevent the formation of a water film on plant surfaces that is essential for pathogen survival and proliferation. The cuticle layer also acts as a structural barrier to prevent the entry of the pathogen into the host to a certain extent. Plants also benefit from the thick and tough walls of epidermal cells, which makes the direct penetration of the pathogen difficult and help plants to resist invaders (Agrios, 1998). In conjunction with this, the deposition of complex polysaccharides such as callose and another complex heteropolymer of cinnamyl alcohols such as lignin around the pathogen infection site are believed to hinder the pathogen by making the cell wall highly resistant to mechanical penetration. Also, they hinder the pathogen entry by restricting the diffusion of toxins and enzymes from the pathogen to the inside of the host and also by inhibiting the flow of water and nutrients from host to pathogen (Vance *et al.*, 1980; Kudlicka and Brown, 1997).

Phytoalexins are toxic antimicrobial substances produced by plants in response to pathogen attack that accumulate around necrotic tissues of both resistant and susceptible tissues (Agrios, 1998). Resistance or tolerance to the pathogen is dependent on the concentration of the phytoalexin around the necrotic area, which may restrict further disease progression (Kuc, 1995). Some of the known phytoalexins are phaseollin in bean, pisatin in pea, glyceollin in soybean, camalexin in *Camelina sativa*, and brassilexin in crucifers (Ebel, 1986).

It is well known that the degree of susceptibility and resistance of a host with respect to various pathogens is an inherited trait (Agrios, 1998). The co-existence of

host plants and their pathogens in nature indicates that the two have been co-evolving (Agrios, 1998). The equilibrium between the virulence changes in the pathogen is maintained by the changes in the resistance of the host and vice versa. Therefore, evolution of both virulence and resistance together can be explained by the gene-for gene concept which is another interesting aspect of incompatible and compatible plant-pathogen interactions (DeWit, 1992). This concept of gene-for-gene interaction was first demonstrated by H. H. Flor in the flax-flax rust (*Melampsora lini)* interaction (Flor, 1956). According to this concept, "for each gene determining resistance in the host, there is a corresponding gene for avirulence in the pathogen with which it specifically interacts". Resistance (R) genes such as *Pto* (Tang *et al.*, 1999), *Cf-9* (Hammond *et al.*, 1998), *N* (Witham *et al.*, 1996), respond specifically to the products of a pathogen's gene (known as avirulence genes) and R-gene-mediated resistance has offered several attractive options for disease control by transferring multiple R-genes simultaneously. It is reported that tightly linked R-genes act synergistically, which might increase the durability of the resistance (Parniske *et al.*, 1997).

The hypersensitive reaction (HR) is a specific form of programmed cell death, which occurs as a defense against different plant pathogens (Gilchrist, 1998; Heath, 1998), typically in gene-for-gene interactions (Heitefuss, 2001). Resistance through the HR is considered to be the result of recognition of the elicitor (produced by the avirulence gene of the pathogen) by a R-gene-coded specific receptor molecule of the host plant (Keen, 1992; Lamb, 1994). Several complex interactions are involved in the HR. For instance, the oxidative burst, which is a rapid production of reactive oxygen species like superoxide anions (O_2^-), hydroxy radicals (OH^-) and hydrogen

peroxide (H_2O_2) plays a crucial role in triggering HR (Lamb and Dixon, 1997). Plants have been engineered (genetically modified organisms) to continuously produce active oxygen species as a strategy to counter pathogen infection. For example, expression of a less active catalase in transgenic tobacco plants led to increased accumulation of H_2O_2 and led to high tolerance to the pathogen (Chamnongpol *et al.*, 1998).

Induced resistance in plants is a physiological "state of enhanced defensive capacity" triggered by specific environmental stimuli and is characterized by the activation of the plant's natural defense systems against subsequent contacts with the pathogen (Van Loon *et al.*, 1998). This strategy has been effectively used by plant species against a broad range of pathogens including fungi, viruses, bacteria, nematodes and even parasitic plants and insect herbivores (Sticher *et al.*, 1997; Benhamou and Nicole, 1999; McDowell and Dangl, 2000; Kessler and Baldwin, 2002). There are two types of induced resistance mechanisms that are well understood, systemic acquired resistance (SAR) and induced systemic resistance (ISR). SAR is a whole-plant response wherein a prior localized exposure to a pathogen results in the entire plant exhibiting resistance to the same pathogen during subsequent exposures. In its classic form, SAR is associated with local and systemic accumulation of salicylic acid (SA) along with the induction of a battery of genes called pathogenesis-related (PR) genes. SAR can be induced upon contact with a range of pathogens and the resistance observed following induction of SAR is also effective against an array of pathogens. On the other hand, ISR is associated with the ability of rhizobacterial strains that promote plant growth to protect against a variety of fungal and bacterial pathogens and is relatively less "broad-spectrum" in nature (Bostock,

2005). ISR does not display accumulation of SA or PR proteins (Pieterse *et al.*, 1996), but instead exhibits a dependence on pathways regulated by jasmonic acid (JA) and ethylene (Knoester *et al.*, 1999; Yan *et al.*, 2002).

1.3. Canola (*Brassica napus* L.) – an introduction

1.3.1. History and origin

Brassica crops are considered as one of the oldest cultivated plants. *Brassica rapa* is mentioned in ancient Sanskrit literature from ca. 1500 BC and seeds of *B. juncea* have been found in archaeological sites dating back to about 2300 BC in India (Prakash, 1980). China also has a long history of rapeseed production and the Chinese word for rapeseed was first recorded about 2500 years ago, with the oldest archaeological discoveries dating back to ca. 5000 BC (Yan, 1990).

Historically, *Brassica rapa* has the widest distribution among the *Brassica* oilseeds. It is estimated that approximately 2000 years ago, the species was distributed from Europe to China and Korea, with the primary center of diversity in the Himalayan region (Hedge, 1976). *B. napus* probably developed in the area where the wild forms of its ancestral species are sympatric, i.e. in the Mediterranean area. Wild forms of *B. napus* are unknown, so it is possible that it originated in cultivation (Sovero, 1993). Production of oilseed *B. napus* likely started in Europe during the middle-ages; *B. napus* was introduced to Asia during the 19th century. The present

Chinese and Japanese germplasm was developed by crossing European *B. napus* with different indigenous *B. rapa* cultivars (Shiga, 1970).

1.3.2. Taxonomy

B. napus L. and *B. rapa* L. belong to the family Cruciferae (Brassicaceae), also known as the mustard family. The four most widely cultivated species of the plant genus *Brassica*, such as *B. juncea*, *B. napus*, *B. oleracea*, and *B. rapa* are highly polymorphic including oilseed crops, root crops, and vegetables such as Chinese cabbage, broccoli, and brussel sprouts.

The relationships among the cultivated species were explained by cytological work carried by Morinaga (1934). According to his hypothesis, the high chromosome number species *B. napus* (2n = 38, AACC), *B. juncea* (2n = 36, AABB), and *B. carinata* (2n = 34, BBCC) are amphidiploids combining in pairs the chromosome sets of the low chromosome number species *B. nigra* (2n = 16, BB), *B. oleracea* (2n = 18, CC), and *B. rapa* (2n = 20, AA). This hypothesis was verified by U (1935) with the successful re-synthesis of *B. napus* and explained that many *Brassica* species were derived from three ancestral genomes denoted by the letters AA, BB and CC (U, 1935). Frandsen (1943; 1947) accomplished the re-synthesis of *B. juncea* and *B. carinata*. It was also suggested that the low chromosome number species may have developed from ancestral species with even lower chromosome numbers (Robbelen, 1960).

1.3.3. Uses

"Canola" is a name applied to edible oilseed rape, derived from two *Brassica* species, *B. napus* L. and *B. rapa* L. Rapeseed oil was primarily used in Europe and Asia as oil for lighting lamps. With the passage of time and development of improved processing techniques, it was steadily used as cooking oil and as a lubricant in steam engines (Robbelen *et al.*, 1989). Rapeseed oil was however unpopular as an edible oil due to a few undesirable characteristics. Chief among them was the high content of erucic acid (22:1) as well as its unpleasant taste and greenish color. Rapeseed meal was also unattractive as a cattle feed due to high levels of sharp-tasting compounds called glucosinolates. The Canadian plant breeder, Dr. Baldur Stefansson, developed a commercial variety of rapeseed in 1974 which was low in both the erucic acid and glucosinolates. This variety was dubbed "canola" (a contraction of "Canadian Oil Low Acid") and was adopted as a registered trademark of the Western Canadian Canola Association to describe "double-low" varieties, which refer to cultivars of oilseed rape that produce seed oils with less than 2% erucic acid and the solid component of seed with less than 30 μmol of aliphatic glucosinolates per gram (http://www.canola-council.org).

Canola oil contains only traces of erucic acid, 5 % to 8 % saturated fats, 60 % to 65 % monosaturated fats, and 30 % to 35 % polyunsaturated fats (http://www.canola-council.org). As a result, canola oil is being widely used as cooking oil, salad oil, and in making margarine. It has also caught the eye of health-conscious consumers because of the lowest saturated fat content among all major

edible vegetable oils (including sunflower, corn and peanut). Canola oil is also a promising candidate for the manufacture of biodiesel, a renewable alternative to fossil fuels and canola meal serves as a nutritious livestock feed.

1.3.4. Economic importance

Oilseed rape is now the second largest oilseed crop in the world, providing 13% of the world's supply (Raymer, 2002). The world's commerce in oilseeds is largely supplied by two species, *B. napus* L. (Argentine type) and *B. rapa* L (Polish type). Argentine varieties have a higher yield potential, are taller and have higher oil content than Polish varieties. Seeds of these species commonly contain 40 % or more oil and produce meals with 35 to 40 % protein. Canola is produced extensively in Europe, Canada, Asia, Australia, and to a more limited extent in the United States (Raymer, 2002). Primary production areas of canola (low erucic acid rapeseed) in Canada are the prairie provinces of Alberta, Manitoba and Saskatchewan.

Canola has been considered as one of the major cash crops in North America and between 7 and 10 million metric tons of canola seed per year is produced in Canada and United States. The canola industry contributes more than $6 billion annually to the Canadian economy (http://www.canola-council.org/). Over 70 percent of Canada's canola crop is exported around the world (e.g. United States, Japan, Mexico, China, Pakistan), which includes 3 to 4 million metric tons of the seed, 706,000 metric tons of canola oil and 1.15 million metric tons of canola meal annually (www.canola-council.org).

1.4. Important diseases of canola

Several kinds of pests, weeds and fungal agents are responsible for reduction in canola yield, affecting the canola industry. Some of the common bacterial diseases such as bacterial blackrot caused by *Xanthomonas campestris*, bacterial pod rot caused by *Pseudomonas syringae* and bacterial soft-rot caused by *Erwinia carotovora* Virus-infected diseases of canola are crinkle caused by Turnip Crinkle Virus, mosaic caused by Cauliflower Mosaic Virus and yellows by Beet Western Yellows virus. Most important diseases of canola are fungal diseases, which include Phoma stem canker (blackleg) disease caused by *Leptosphaeria maculans*, Alternaria blackspot by *Alternaria brassicae*, clubroot by *Plasmodiophora brassicae*, Fusarium wilt caused by *Fusarium oxysporum* and stem rot of canola by *Sclerotinia sclerotiorum*. These are considered to be economically significant diseases of canola. In 2004, an overall mean percent of disease incidence was recorded as 4.1% (blackleg), 2.1% (black spot) and 12.4% (stem rot) in Alberta province (Dmytriw and Lange, 2005), which still counted as a significant amount of canola crop loss. The interaction of canola with *L. maculans* and *A. brassicae,* both of which belong to the same order, Pleosporales, is described in subsequent sections.

1.4.1. Phoma stem canker disease of canola

Blackleg disease of canola is one of the most economically important diseases in both oilseed rape and vegetable crucifers worldwide, responsible for losses in oilseed rape yield amounting to more than CDN $30 million per year (Taylor *et al.*, 1995). West *et al.*, (2000) proposed the term phoma stem canker to cover all symptoms of stems and leaves such as blackleg, crown canker and phoma stem lesions. This disease is also known as blackleg because stem lesions may appear as a general blackening at the base. Stem cankers caused by *L. maculans* are important because they girdle the stems, resulting in lodging and death of the plants, causing significant loss to the crop (Gabrielson, 1983). Virulent pathotypes of the pathogen have been causing serious problems on canola in Canada (Petrie, 1986).

Severe yield losses of up to 90%, caused by stem canker disease were observed in Wisconsin cabbage-growing areas (Henderson 1918). However, blackleg disease became a great economic concern only with the success of oilseed rape or canola, as an important oilseed crop in various parts of the world. Also, until 1950, blackleg disease was not considered to be an important disease but major epidemics in France in 1950 and 1966; in Australia in 1972 and in England in 1977, which led to 50% yield losses and brought this disease into the limelight (Gugel and Petrie, 1992). After identification of this disease in 1975 in Canada, disease incidence was found to increase by a factor of 10 between 1978 and 1981 (Juska *et al.*, 1997). About 65% of the fields in Saskatchewan were identified with the presence of the pathogen by 1986 (Jesperson, 1989) and it subsequently spread to Ontario in 1986, Manitoba in 1987 and Alberta in 1988 (Gugel and Petrie, 1992). Regions of Manitoba and Alberta suffered about 10% of yield losses due to blackleg in 1987 and 1988 respectively (Gugel and

Petrie, 1992). Losses of $50 million (Canadian) per year due to blackleg disease were estimated by the Canola Council of Canada in 1997 (Juska *et al.*, 1997). Australia suffered significant losses of A$ 18.6 and A$ 49.4 million in oilseed crop in 1998 and 1999, respectively, as a result of this disease (Khangura and Barbetti, 2001). Recently, up to 70% yield loss was reported in Central Mexico due to the presence of an aggressive isolate of *L. maculans* belongs to pathogenicity group 2 (PG2) (Moreno-Rico *et al.*, 2002).

1.4.1.1. Origin and taxonomy

As indicated previously, *Leptosphaeria maculans* (Desm.) Ces. & de Not. (anamorph *Phoma lingam* Tode ex Fr.) is the causal agent of blackleg disease of oilseed rape, which leads to extensive canola crop losses worldwide (West *et al.*, 2001). Tode (1791), first recorded association of this fungal species with dried stems of red cabbage (*B. oleracea*) and it was named as *Sphaeria lingam* at that time (cited in Henderson, 1918). Later in 1849, this fungus was recognized as a pathogen of cabbage by Desmazieres and renamed as *Phoma lingam*. *Leptosphaeria maculans* (Desm.) Ces. & de Not. was suggested as the sexual stage of *P. lingam* by Tulasne and Tulasne in 1863.

Leptosphaeria maculans is an ascomycete fungus, belonging to the largest order, the Pleosporales of the class Dothideomycetes (Rouxel and Balesdent, 2005). Koch *et al.* (1991) classified isolates of *L. maculans* as non-aggressive (pathogenicity group; PG1) and aggressive (PG2, 3 and 4) based on the cotyledon reactions of the

three *B. napus* cultivars that are Westar, Quinta and Glacier. They also differentiated these groups on the basis of their ability to produce phytotoxin such as sirodesmins *in vitro* by the aggressive isolates suggesting that sirodesmins contribute to pathogenicity. Pathogenicity tests based on an interaction phenotype (IP) aided the determination of pathogenicity groups (PG) of the isolates of *L. maculans* with their reactions to differential cultivars (Mengistu *et al.*, 1991). Highly virulent PG2, 3 & 4 (A-group), which are responsible for the highly damaging stem canker and a weakly virulent group PG1 (B-group) causes less damaging phoma stem lesions (Williams and Fitt, 1999; Rouxel *et al.*, 2004). Isolates of PG1 have been classified as a separate species *Leptosphaeria biglobosa* due to their non-aggressive nature on Westar, Quinta and Glacier (Shoemaker and Brun, 2001).

L. maculans and *L. biglobosa*, two closely related species, were distinguished on the basis of morphological differences of pseudothecia (Shoemaker and Brun, 2001) whereas seven subspecies within these 2 species were resolved on the basis of molecular and biochemical differences (Mendes-Pereira *et al.*, 2003). Most of the isolates occurring on *Brassica* species are related to *L. maculans* and *L. biglobosa*, which are distributed worldwide including Canada, Europe and USA (Mendes-Pereira *et al.*, 2003). These two species are known to share similar host-range and sexually reproduce on host tissues (Jedryczka *et al.*, 1999; West *et al.*, 2002). However, *L. maculans* has been found to be predominantly responsible for the early leaf spots which are associated with damaging basal stem canker (West *et al.*, 2002).

L. biglobosa (PG1) is associated with upper stem lesions (Huang *et al.*, 2005), also known to be weakly virulent or avirulent as compared to *L. maculans* (PG2, PG3

and PG4), which is responsible for damaging stem base canker (West *et al.*, 2001). Reduced disease severity was observed when *L. biglobosa* was pre- or co-inoculated with virulent isolates of *L. maculans* on susceptible cultivars of *B. napus* (Chen and Fernando, 2006). *L. maculans* was reported to occur on various crucifers, including cruciferous weeds (Petrie, 1969), but many of the isolates obtained from the cruciferous weeds were different subspecies of *L. biglobosa* (Mendes-Pereira *et al.*, 2003). With respect to *L. maculans* species, recent reports indicate that this fungus can infect various cruciferous taxa in the field and could be isolated from species such as *Raphanus sativus, Sinapis alba, R. raphanistrum* etc. (Li *et al.*, 2005).

1.4.1.2 Characteristics of the pathogen

L. maculans is a heterothallic pathogen that outcrosses prolifically on canola stubble (Boker *et al.*, 1975). It has a mixed reproductive system, with one sexual cycle per growing season and an asexual cycle during the other phase. Because of having such a reproduction system, it may pose greater risk of breaking down resistance genes by increasing the frequency of isolates through recombination (in sexual reproduction) and clonal reproduction (in asexual cycle) to overcome host resistance (McDonald and Linde, 2002). This pathogen is responsible for causing cotyledon and leaf lesions during the vegetative phase of the host plant whereas it causes crown canker in the adult phase of the plant, which leads to drastic yield losses (Barbetti, 1975, Boker *et al.*, 1975). Disease-spread is supported by warm temperature and high humidity; spores can travel by air at least 2 km and inoculum on stubble can

remain viable for at least 5 years (Hall, 1992). *L. maculans* is saprophytic on stem residues and spores produced through sexual mating can infect cotyledons and leaves by penetrating the tissues through stomata and wounds (Rouxel and Balesdent, 2005). In most cases, airborne ascospores (sexual) are considered to be the primary source of inoculum in canola crop (Brunin and Lacoste, 1970). Pycnidiospores (asexual), produced by pycnidia, act as a secondary source of inoculum (Hammond *et al.*, 1985) and are required in higher concentrations than ascospores to cause disease symptoms (Wood and Barbetti, 1977). Li *et al.* (2006) made an effort to evaluate the potential of pycnidiospores to cause disease in the presence of ascospores by paired co-inoculation on *B. napus* cotyledons and concluded the polycyclic nature of blackleg disease epidemics throughout the cropping season.

1.4.1.3. Disease cycle, signs and symptoms

Blackleg disease of canola is particularly difficult to control due to the life cycle of the pathogen. The blackleg fungus can over-winter on infected canola residue and in infected seed (Jacobsen & Williams, 1971). In the spring, the fungus produces fruiting bodies, called pseudothecia, on infected canola residue (Petrie, 1978; Boker *et al.*, 1975). Pseudothecia release small airborne microscopic spores, called ascospores, resulting in long distance dispersal on newly planted canola crops and causing leaf lesions from the seedling stage onward. Most likely, development of basal stem canker is favored by an early infection and leads to severe yield losses. Pseudothecia

may continue to be produced on infected residue for two more years, or until the infected residue breaks down.

After harvest, the blackleg fungus abundantly produces another type of fruiting body called a pycnidium, which exudes conidia (West *et al.,* 2000). These spores disperse short distances by rain splash and cause secondary infection within a crop. These secondary lesions are commonly observed in fields that received injury from hail damage. The fungus can move systemically in the plant's vascular tissue to the stem base through infected leaves and directly infect the wounded stems. Blackleg infections may occur on cotyledons (Boker *et al.*, 1975), leaves (Hammond *et al.*, 1985; Barbetti and Khangura, 2000), hypocotyls and stems (Kharbanda, 1993) and, as a result, canola is susceptible to blackleg infection from the seedling to pod-set stages.

Lesions occurring on the leaves are pale green spots, dirty white, round to irregularly shaped, containing numerous small, black pycnidia, which are the spore-bearing structures of the fungus. Pycnidia appear as tiny round specks, which produce conidia (West *et al.*, 2001). Hypocotyl infection produces a constriction in the stem above the ground (Kharbanda, 1993; Barbetti and Khangura, 1999). On stems, blackleg lesions are usually found at the base or at points of leaf attachment with often a distinct dark brown and purple margin (Hammond *et al.,* 1985). Numerous pycnidia form in the center of the lesion. Severe infection usually results in a dry rot or canker at the base of the stem (Paul and Rawlinson, 1992). The stem becomes girdled and as plants ripen prematurely, the crop is more likely to lodge. Seeds may be shriveled and pods shatter easily at harvest, resulting in seed loss (Davies, 1986; Petrie and

Vanterpool, 1974). Recently, root infection has also been reported by Sosnowski *et al.* (2001), which suggested that disease could be soil-borne as plants with infected lateral roots and hypocotyls showed stem and crown cankers. Fungal proliferation within root tissue was studied in detail recently by transforming an isolate of *L. maculans* with Green Fluorescent Protein (GFP; Sprague *et al.*, 2007). They observed that *L. maculans* can directly infect an intact root and can also proliferate within the root tissue following foliar inoculation (Sprague *et al.*, 2007).

1.4.1.4. **Plant Responses to *L. maculans***

As described in the general introduction, phytoalexin production in plants helps to combat diseases. Crucifers are known to accumulate sulfur containing phytoalexins. For example, brassilexin isolated from *B. juncea* was found to have antifungal activity to *L. maculans* (Devys *et al.*, 1988). Pedras *et al.* (1998) reported that both phytoalexins brassinin and cyclobrassinin are biosynthetic precursors of brassilexin. Wasalexins isolated from *Wasabia japonica*, syn *Eutrema wasabi*, plants resistant to *L. maculans* were found to have potent antifungal activity against *L. maculans* (Pedras *et al.*, 1999). On the other hand, pathogens are capable of detoxifying these phytoalexins by converting them into harmless metabolites (Pedras *et al.*, 2007). For example, it has been reported that *L. maculans* can detoxify the most potent antifungal phytoalexins produced by crucifer plants such as brassilexin and sinalexin (Pedras and Suchy, 2005). However, camalexin isolated from *C. sativa* is not metabolized by *L. maculans* (Pedras *et al.*, 1998).

Induced resistance in plants is triggered by the onset of disease (Kessmann *et al.*, 1994). Systemic acquired resistance was found to be induced by the co-infection of weakly virulent and highly virulent isolates of *L. maculans* in *B. napus* (Mahuku *et al.*, 1996). They also found the reduction in the lesion size caused by inoculation with the weakly virulent isolate followed by highly virulent one as compared to lesion caused by inoculation with highly virulent isolate only.

A variety of enzymes active against plant wall constituents such as pectin, cellulose, xyloglucan are often produced by phytopathogenic fungi. *L. maculans* possesses cellulase and polygalacturonase activities in culture (Hassan *et al.*, 1991) and in stem lesions on *B. napus* (Easton and Rossall, 1985). These enzymes may produce oligosaccharide elicitors/ suppressors of host-plant defenses (Boudart *et al.*, 1998, Moerschbacher *et al.*, 1999).

Presently, disease control relies mainly on the use of disease-resistant cultivars (Gout *et al.*, 2006). Two types of inheritance of disease resistance have been observed in *B. napus*: polygenic or quantitative (Pilet *et al.*, 1998a and 2001) and monogenic or qualitative resistance (West *et al.*, 2001). Roy (1978) reported a successful transfer of resistance from *B. juncea* to *B. napus* and suggested in 1984 that *B. juncea* resistance came from the B genome (Roy, 1984). Blackleg resistance has been identified in germplasm from Canada, Australia and Europe (Bansal *et al.*, 1994; Brunin and Lacoste, 1970; Cargeeg and Thurling, 1980; Rimmer and Van den Berg, 1992; Stringam *et al.*, 1992). Recently, Christianson *et al.* (2006) constructed the map of the genome of *B. juncea* using restriction fragment length polymorphism (RFLP) and microsatellite markers and they determined the genomic locations of B-genome

derived resistance traits. *Brassica nigra* and *B. carinata* were found to have a high degree of blackleg resistance and were used to generate resistant lines through a breeding program (Rimmer and Van den Berg, 1992; Sacristan and Gerdemann, 1986) but some isolates of *L. maculans* were assessed as virulent on *B. nigra* and *B. juncea* from Australia and France (Purwantara *et al.*, 1998; Brun *et al.*, 2001). So far, the resistance of *B. carinata* has not been broken by any isolate of *L. maculans* and therefore, it could be used as a potential source of blackleg resistance for breeding and biotechnological perspectives (Subramanian *et al.*, 2005).

Most research on resistance to *L. maculans* in *B. napus* has focused on genetic identification of resistance genes and factors responsible for quantitative responses (Quantitative Trait Loci; QTL) (Pilet *et al.*, 1998a, 1998b, 2001). Nine avirulence genes of *L. maculans*, (*AvrLm1* to *9;* Ansan-Melayah *et al.*, 1995; Balesdent *et al.*, 2001; 2002; 2005) and their corresponding resistance genes (*Rlm1* to *9;* Delourme *et al.*, 2004; Rouxel and Balesdent, 2005) in *B. napus* have been identified in host-pathogen systems with gene-for-gene interactions. These R- genes could be effective in controlling a disease only in the presence of avirulence allele in the pathogen. However, commercially available cultivars with single effective R-gene experience the rapid breakdown of major gene resistance due to the adaptation of *L. maculans* field populations and such breakdown has been reported in France (Rouxel *et al.*, 2003) and Australia (Li *et al.*, 2003). Gout *et al.*, (2006) assessed the rapid diffusion of novel virulence alleles of French populations of *L. maculans* whenever novel resistance sources were exploited.

Expression of resistance responses such as callose deposition and deformation of hyphae was observed in *B. juncea* upon challenging with avirulent isolate of *L. maculans* (Chen and Howlett, 1996), whereas a hypersensitive response was triggered in *B. napus* in response to an avirulent isolate of *L. maculans* (Roussel *et al.*, 1999). This suggests that resistance responses to *L. maculans* in *B. napus* could be a cumulative effect of many molecular and biochemical pathways.

Subramanian *et al.* (2005) reported proteome-level changes in a *Brassica* line, obtained through an interspecific cross between susceptible *B. napus* and resistant *B. carinata* upon challenging with *L. maculans*. This report revealed the presence of several proteins involved in photosynthetic and metabolic pathways along with some antioxidant enzymes. Wang *et al.* (1999) showed that constitutive expression of pea defense gene DRR206 provides resistance to *B. napus* against PG2 isolate of *L. maculans*. Yu *et al.* (2005) identified two genes (*LepR1* and *LepR2*) for blackleg resistance from two populations of *B. napus* doubled-haploid (DH) lines (resistance was introduced from *B. rapa* subsp. *sylvestris* through plant breeding). Thus, our knowledge about the genes involved in mediating resistance to this pathogen in plants is limited and could benefit from additional studies.

1.4.2. Alternaria blackspot disease of canola

In the western Canadian provinces, *Alternaria brassicae* and *Alternaria raphani* are considered to be the most dangerous and prevalent causal agents of blackspot disease of canola (Clear and Patrick, 1995). Blackspot disease is more

damaging on Polish canola (*B. rapa*) than on Argentine canola (*B. napus*). Alternaria backspot disease is endemic in the northern canola growing regions of the Canadian Prairie Provinces. Yield reductions of up to 36% in canola have been caused by *A. brassicae* (Rude *et al.*, 1994; Verma and Saharan, 1994; Duczek *et al.*, 1998).

1.4.2.1 Origin and taxonomy

As mentioned earlier, blackspot disease on *Brassica* species is caused by *A. brassicae*, a pathogen of several cruciferous plant species. This disease on oilseed rape, cabbage and mustard crops was reported in India (Kadian and Saharan, 1983), in most of the European countries (Gladders, 1987) and in Western Canada (Conn and Tewari, 1990; Berkenkamp and Kirkham, 1991). The *Alternaria* genus was established in 1817 with *A. alternata* as the isolate type and classified into the phylum *Fungi imperfecti* (phylum of fungi without known sexual stages), class Hypomycetes, order Pleosporales (Thomma, 2003). This genus is differentiated from other genera on the basis of its conidial characteristics such as conidiophore apex and basal end of immature conidia (Simmons, 1995). Even within the genus *Alternaria*, species are also defined upon conidium characteristics. Over 100 species of *Alternaria* have been described worldwide so far (Simmons, 1992).

1.4.2.2. Characteristics of the pathogen

Alternaria brassicae (Berk.) Sacc. is a species with small dark spores forming colonies, which sporulate abundantly and may produce chains of more than ten conidia (Rangel, 1945). White light of relatively low intensity was reported to be inhibitory to sporulation of this fungus (Humpherson-Johns and Phelps, 1989). *Alternaria* species possess two main features, production of melanin, especially in the spores and production of host-selective toxins (Thomma, 2003). Recently, reduced conidial size and septal number in *A. alternata* after the disruption of a melanin biosynthesis gene was reported, which suggests the active involvement of melanin in conidial development (Kawamura *et al.*, 1999).

Melanin is a high molecular weight pigment, with a dark brown to black color, produced by organisms ranging from animals and plants to micro-organisms. The production of melanin pigment is conserved among *Alternaria* species. Oxidative polymerization of phenolic and indolic compounds leads to the production of melanins (Thomma, 2003). They are aromatic ring and hydroxyl group containing molecules (Bell and Wheeler, 1986) and also contain stable populations of free radicals (Enochs *et al.*, 1993). Melanin pigment is produced by *Alternaria* from the monomeric precursor 1, 8-dihydroxynaphthalene (DHN), through the pentaketide pathway (Kimura and Tsuge, 1993). Melanins play a very important role in virulence along with the role in conidial development (Kawamura *et al.*, 1999). These pigments protect fungi against environmental stress and unfavorable conditions such as extreme temperature and UV radiation (Kawamura *et al.*, 1999; Lockwood, 1960; Rehnstrom and Free, 1996).

Bains and Tewari (1987) identified a phytotoxin named Destruxin B from *A. brassicae* and considered it to be a host-specific toxin. Later, it was found to cause necrotic and chlorotic lesions on both host and non-host plants such as non-crucifer species and monocotyledonous plants. However, non-host species showed less sensitivity to destruxin B than host species (Buchwaldt and Green, 1992). Two more destruxins named homodestruxin B and desmethyl destruxin B from *A. brassicae* were isolated and identified (Ayer and Pena-Rodriguez, 1987). In addition to destruxin B and homodestruxin B, destruxin B_2 was also isolated from *A. brassicae* and found to be phytotoxic (Buchwaldt and Jensen, 1991).

1.4.2.3. Disease cycle, signs and symptoms

Sporulation of *A. brassicae* on naturally infected leaf discs of oilseed rape requires humidity equal to or higher than 91.5% and an optimum temperature of 18-24°C (Humpherson-Johns and Phelps, 1989). *Alternaria* species produce secondary conidia, which allow these species additional opportunities to overcome unfavorable conditions (Alexopoulos, 1966; Taber, 1964; Tsuneda and Skoropad, 1977). Infected debris of *Brassica* crops remaining on the ground after harvest may provide a source of inoculum, which may be responsible for the disease spread within and between crops (Humpherson-Jones, 1989). This pathogen also occurs on cruciferous weeds and forage brassica (Humpherson-Jones and Hocart, 1983). An injury to the plant is not necessary for infection with *A. brassicae*, because of its highly virulent nature. Seeds may carry pathogens both as spores and as latent mycelium (Rangel, 1945).

A. brassicae can infect canola plants in all stages of growth. Rapid and more severe infection was observed in older leaves than younger ones. An explanation for this could be that *A. brassicae* sporulates on decaying parts in contact with the soil; therefore, spores are splashed by rain drops over the lower leaves rather than younger ones (Rangel, 1945).

Alternaria brassicae is a foliar pathogen, causes slow destruction of the host tissues through the reduction in photosynthetic potential. It does not affect water and nutrient transport in the plant, because it does not infect roots or transport vessels (Rotem, 1994). It resides in the centre of the lesion, surrounded by a chlorotic halo. This zone is formed by the diffusion of fungal metabolites such as toxins (Agarwal *et al.*, 1997; Tewari 1983). Therefore, symptoms of Alternaria blackspot of canola range from necrotic lesions through premature ripening of pods and pod shattering to shrunken seeds (Dorrance, 1994). Under favorable conditions, spore produces one or more germ tubes, which subsequently penetrates stomata, cuticle and wounds directly (Rotem, 1994).

1.4.2.4. Plant responses to *A. brassicae*

There are limited reports of resistance to this pathogen in plant species. For example, Conn *et al.* (1988) showed that the leaves of *Camelina sativa* (false flax) are resistant to *A. brassicae*. This resistance was attributed to the production of the phytoalexin camalexin (isolated from *C. sativa*) with fungitoxic activity against *A. brassicae* (Browne *et al.*, 1991). *A. brassicae* was not able to metabolize camalexin,

which also appeared to interfere with the production of the phytotoxin destruxin B by this pathogen (Pedras *et al.*, 1998). Resistance within *Brassica* species against *A. brassicae* is not known yet but other members of the cruciferae family are known for their resistance to blackspot disease, such as *Sinapis alba, Capsella bursa-pastoris* and *Eruca sativa* (Conn *et al.*, 1988; Conn and Tewari, 1986).

Sinapis alba was found to metabolize destruxin B to a less toxic product, faster than susceptible species through hydroxylation and glucosylation reactions (Pedras *et al.*, 2001). *C. bursa-pastoris* and *E. sativa* may interfere with the biosynthesis of dextruxin B and may also detoxify it thereby rendering these plants species resistant to this pathogen (Pedras *et al.*, 2003). It has also been reported that systemic resistance in Chinese cabbage against *A. brassicae* was induced by the root endophytic fungus *Heteroconium chaetospira* (Morita *et al.*, 2003). Recently, overexpression of a pathogenesis-related (PR) protein glucanase, which hydrolyzes glucan (a major component of the fungal cell wall) in *B. juncea* led to the delayed onset of the disease and the transgenic plants were observed to arrest 15-54% of hyphal growth of *A. brassicae* (Mondal *et al.*, 2007).

1.4.2.5. Pathogenic determinants

Little is known about the pathogenic determinants produced by *A. brassicae,* which helps in penetration and colonization of plant tissues. Recently, two physically clustered genes, encoding nonribosomal peptide synthetase (NRPS) and ATP-binding cassette (ABC) transporter were identified on the *A. brassicae* genome (Guillemette *et*

al., 2004). These genes may be involved in phytotoxin synthesis or in synthesis of siderophores, which pathogens use to take iron from the host during infection (Guillemette *et al.*, 2004; Renshaw *et al.*, 2002). The phytohormone cytokinin (zeatin) was also suspected to be involved in the establishment of blackspot disease of *B. juncea* (Garg *et al.*, 1999). They proposed the possible association of phytotoxins and phytohormones in the cell signal transduction pathway of pathogenesis in an antagonistic manner.

1.4.3. Management of blackleg and blackspot diseases

Cultural control of plant diseases involves disease avoidance, pathogen exclusion, pathogen eradication and disease resistance (Kharbanda and Tewari, 1996). Infected *Brassica* seeds carry blackleg and blackspot diseases to uninfected areas (Petrie, 1979; Chahal and Kang, 1979; Rotem, 1994). Seed health and removal of infected seeds is one of the measures of cultural practices. However, exclusion of seed-borne inoculum is not effective to control these diseases where spore inoculum is abundant (Kharbanda and Tewari, 1996). Time adjustment of seeding to avoid high levels of inoculum or conditions conducive for disease development can also contribute to management of diseases. In Canada, it has little influence on disease severity, especially blackleg due to the presence of ascospores throughout the season (Gugel and Petrie, 1992; Kharbanda, 1992; Petrie, 1986). Infected seed was found as a primary source of blackleg introduction (Kharbanda, 1992). It was noted that seed-borne inoculum helped in the introduction and development of the blackleg disease in

certain counties of Ontario (Hall *et al.*, 1993). Reduction in the disease severity was recorded due to crop rotation providing at least a 3-year gap between canola crops (Petrie, 1986). *L. maculans* can survive in infected plant stubble for more than 5 years but the most destructive infections occurred from 2-3 years old stubble (Petrie, 1979). In order to control blackleg by employing a cultural approach, crop debris should be buried in the fall before crop rotation (Gladders and Musa, 1980, Kolte, 1985).

Although cultural practices such as stubble management and crop-rotation help in the reduction of disease incidences, disease cannot be prevented (West *et al.*, 2001). Fungicides such as carbathin, thiram and iprodione are currently registered as seed treatments in Canada (West *et al.*, 1999). Different combinations of fungicides are used to control phoma stem canker. For instance, flutriafol applied to fertilizer granules can protect young seedlings more than seed treatment alone for a longer time, but serious losses from the disease can still occur in the crops grown with fungicide-treated fertilizer granules (Barbetti and Khangura, 1999). Therefore, it is recommended that fungicide application be integrated with other management techniques and their use is considered unprofitable where crop yields are low (West *et al.*, 2001). Fungicides found to have low eradicant activity and are only effective for limited period due to their degradation, leaf expansion and formation of new untreated leaves (West *et al.*, 2000). Flooding and swathing have also been advocated for disease management (Moore, 1949). Flooding can be effective under special circumstance as it is expensive and also depends on the availability of water-source (Kommedahl and Todd, 1991).

Biological control involves the use of specific micro-organisms that are natural predators, parasites or pathogens in controlling pests/ pathogens by disrupting their ecological status. The Bird's nest fungi, *Cyathus striatus* and *C. olla* decreased the stubble food base for *L. maculans* survival, reduced the available inoculum for further spread, and have been investigated as potential biological control agents (Shinners and Tewari, 1997). Kharbanda *et al.* (1999) reported two antifungal peptides produced by *Paenibacillus poltmyxa* PKB1 bacterium that decrease *L. maculans* growth in culture, leaves, stems and stubble.

Cultural practices may reduce the disease incidences to a certain extent but to make rapid and remarkable progress in preventing plant diseases, there is a need of advancement in our knowledge of fundamental principles as well as details using molecular biology based techniques related to the specific host-pathogen interaction. Two bacterial strains such as *Bacillus endophyticus* and *B. amyloliquefaciens* have also been lately found use as potential biocontrol agents against various disease causing fungal pathogens infecting canola including *L. maculans*, *A. brassicae*, *B. cinerea* and *Verticillium longisporum* (Danielsson *et al.*, 2007).

1.5. Proteomics as a tool to gain insight into plant-pathogen interactions

With the advent of various advanced genomics-based methods, it has become possible to analyze the stress-induced expression of thousands of genes simultaneously (Chee *et al.*, 1996; Yershov *et al.*, 1996) but large genetic variability of gene expression is still a limitation. Proteins expressed from the genes are the entities

which regulate most cellular activities and the presence and absence of a particular gene does not always correlate with the expression of a particular protein in a cell. Proteome analysis represents an essential complement for studying the biological consequences of this variability since it provides information on post-translational modifications, which may occur on expressed proteins (Pandey and Mann, 2000). Therefore, proteomics seems to be relevant for the study of relationships between genotype and phenotype.

In order to understand the biological processes of an organism, it is essential to have knowledge of proteins, which are essential biological determinants of plant and pathogen phenotype. The term "proteome" was first coined by Marc Wilkins in 1995, which describes "the protein complement of the genome" (Wilkins *et al.,* 1995). Proteomics and its associated key technological innovations are over three decades old now (Wilkins *et al.,* 1995; Wasinger *et al.,* 1995) and transition from analyzing one or few proteins at a time to analyzing the entire proteome consisting of thousands of proteins has been achieved (Washburn *et al.,* 2001; Gygi *et al.,* 1999). Proteome-level analyses have been introduced to identify proteins instrumental to different plant responses such as physiological studies due to external sources, at various developmental stages (Finnie *et al.,* 2002) and under different environmental stresses.

Proteomics is basically a large-scale analysis of proteins and this technology is based on the combination of two-dimensional electrophoresis (2-DE) and mass spectrometry identification methods along with others tools available to study the proteome of an organism (Gorg *et al.*, 2004). 2-DE is a powerful means of generating analytical data (Washburn *et al.*, 2001; Gygi *et al.*, 1999; Ross *et al.*, 2004), along

with mass spectrometry. 2-DE techniques have been used to understand the gradation of the proteome in the biological systems and comparisons between two or more samples (Wilkins *et al.*, 2006) by discovering differentially expressed proteins clearly and reproducibly. During the last decade, proteomics has succeeded in identifying the proteins associated with the development and functioning of both plants and their pathogens. It is not only used to identify the changes in protein levels of the plant host infected by their pathogenic counterparts but also to characterize the extracellular and cellular virulence and pathogenicity factors produced by pathogens (Padliya and Cooper, 2006; Kav *et al.*, 2007).

There are both gel-based and gel-free proteomic techniques available for such studies (Baggerman *et al.*, 2005; Lambert *et al.*, 2005). Gel-based techniques include 2-DE and Fluorescence 2-D Difference in-Gel Electrophoresis (DIGE). 2-DE was first described in 1975 and allows the separation of thousands of proteins in a single experiment on the basis of two important parameters: according to their charge by isoelectric focusing in the first dimension, and, to their molecular weight (MW), by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension (O'Farrell, 1975; Gorg *et al.*, 2004). Analysis of 2-DE gels provides several pieces of information about the hundreds of proteins that are visualized including the quantity, MW, pI as well as post-translational modifications (PTMs Gorg *et al.*, 2004; Wittmann- Liebold *et al.*, 2006).

On the other hand, DIGE overcomes the limitation of gel-to-gel variation while comparing images from at least two gels (Van den Bergh and Arckens, 2005). This technique includes, covalent labeling of each sample with a different set of

fluorophores (Cy2, Cy3 and Cy5), and mixing together followed by separation on the same 2-DE gel (Unlu *et al.*, 1997). The resulting images are then analyzed by software such as DeCyder, designed specifically for 2-D DIGE analysis (Marouga *et al.*, 2005). Some of the important gel-free techniques, which aid quantitative measurement of proteins are isotope-coded affinity tag (ICAT; Gygi *et al.*, 1999); multidimensional protein identification technology (MudPIT; Washburn *et al.*, 2001); isobaric tagging for relative and absolute quantitation (iTRAQ; Ross *et al.*, 2004); and various techniques for functional analysis of proteome and protein-protein interactions such as protein microarrays (MacBeath, 2002; reviewed by Kav *et al.*, 2007). The specific proteins selected from the separation by 2-DE are identified using mass spectrometry. Mass spectrometers consist of an ion source, the mass analyzer and an ion detection system which generates raw data by measuring the mass-to-charge ratio (m/z) of the peptides (Mann *et al.*, 2001; Glish and Vachet, 2003). In the case of gel-free techniques such as ICAT and MudPIT, protein samples are directly analyzed by MS, whereas in gel-based approaches including 2-DE and 2-D DIGE, the protein spots are first excised from the gel and then digested with trypsin. The resulting peptides are then separated by liquid chromatography (LC) or directly analyzed by MS. These experimentally-derived peptide masses are correlated with the peptide fingerprints of known proteins in the databases using search engines (e.g. Mascot, Sequest).

Proteomics-based techniques provide a novel approach to study plant resistance mechanisms against biotic (Zhou *et al.*, 2005; Wang *et al.*, 2005) as well as abiotic stresses (Kav *et al.*, 2004). Kav *et al.* (2007) provided a detailed review about the application of proteomics in the investigation of the interaction between plant and

microbes as well as for the detection of phytopathogens. This review summarized the updates on most of the significant studies conducted using proteomics as a tool to understand the interaction of plants with different microbial agents such as fungal, bacterial, viral, nematodal, oomycete and myccorhizal and rhizobial symbioses. Proteomics-based approaches have been adopted to study several biotic stresses in order to identify proteins from rice leaf in response to the avirulent and devastating virulent races of rice blast fungus, *Magnaporthe grisea* (Kim *et al.*, 2004). Levels of receptor-like protein kinases, ß-1,3-glucanases, thaumatin-like protein, probenazole-inducible protein and rice pathogenesis-related (PR) 10 proteins were observed to be higher in plant protein extracts in an incompatible interaction compared to the compatible interaction, suggesting a possible role for these proteins in mediating tolerance to the pathogen (Kim *et al.*, 2004). Similarly, the responses of wheat to the fungal pathogen *F. graminearum* were characterized using 2-DE and LC-MS/MS, which revealed the differential expression of several antioxidant proteins and pathogenesis-related proteins (PR-2) in a resistant cultivar as compared to a susceptible cultivar (Zhou *et al.*, 2005). Another study reported protein profiles of a resistant wheat cultivar, challenged by the *F. graminearum* at different time-points post infection. Many proteins involved in photosynthesis and carbon metabolism were found in this host-pathogen interaction with a steady up-regulation in expression of numerous stress defense-related proteins from 6 to 24 hours post-inoculation was observed (Wang *et al.*, 2005). Proteome-level investigation of blackleg resistant *Brassica* line in response to the fungal pathogen *L. maculans* has been recently reported (Subramanian *et al.*, 2005). The authors of this study found several

antioxidant enzymes, such as peroxiredoxin and dehydroascorbate reductase along with proteins involved in photosynthetic and nitrogen metabolism to be up-regulated in the resistant line compared to the susceptible line, suggesting the possibility of a cumulative effect of different proteins involved in several mechanisms responsible for the observed susceptibility or tolerance to this pathogen (Subramanian *et al.*, 2005).

Proteomics based analysis has also been exploited in deciphering the mechanisms involved in the interaction between plants and their parasitic counterparts. For example, in order to understand the mechanisms involved in pea resistance to the parasitic plant, *Orobanche crenata* (Castillejo *et al.*, 2004), in various pea varieties differing in their susceptibility to the *O. crenata,* proteome-level analysis was carried out. It revealed proteins involved in carbohydrate and nitrogen metabolism were found to be induced as defense proteins in a resistant genotype in response to this parasitic plant (Castillejo *et al.,* 2004). The root protein profiles of *Medicago truncatula* after infection with an oomycete *Aphanomyces euteiches* were analyzed during a time course experiment using 2-DE (Colditz *et al.,* 2004). Similarly, two lines of *Lycopersicon hirsutum* harbouring two different quantitative trait loci (QTL) that control resistance to bacterial canker through distinct mechanisms were compared using a proteomics-based approach (Coaker *et al.*, 2004). Mathesius *et al.* (2003) used a proteomics-based approach to detect the *M. truncatula* responses to the *N*-acyl homoserine lactone (AHL) signals from the pathogenic bacterium *Pseudomonas aeruginosa.*

Along with its involvement in biotic and abiotic stresses, use of proteomics also succeeded in the identification of proteins associated in mycorrhizal and rhizobial

symbioses (Bestel-Corre *et al.*, 2004). Some of the most recent studies include the proteome-level analysis to understand the interaction between a series of barley genotypes with varying resistance to fusarium head blight caused by *Fusarium graminearum* (Geddes *et al.*, 2008). They identified proteins associated with metabolism, oxidative burst and oxidative stress responses. Similarly, proteins of plasma membrane components of rice involved in the early defense responses to the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* were identified using 2-dimensional electrophoresis (Chen *et al.*, 2007). These proteins include H^+-ATPase, protein phosphatase, hypersensitive-induced response protein, prohibitin, zinc finger and C2 domain protein, universal stress protein, and heat shock protein.

Proteomics-based technologies have also been utilized to detect and identify phytopathogens in the study of plant diseases. There are significant discoveries being made using a proteomics-based approach in the studies of the secretome as well as whole proteome of phytopathogens such as bacteria and fungi. This descriptive explanation of these studies was reviewed by Kav *et al.* (2007) in their latest review. Bacterial secreted protein profiles (secretome) of *Erwinia chrysanthemi* and *Xanthomonas campestris* pv. *campestris* were characterized using 2-DE and MS (Kazemi-Pour *et al.*, 2004; Watt *et al.*, 2005). Furthermore, the whole bacterial cell proteome as well as secretome of *Xylella fastidiosa* were analyzed using a proteomics-based strategy which resulted in the identification of several different proteins involved in cellular adhesion systems (Smolka *et al.*, 2003). Some of the fungal pathogens such as *Magnaporthe grisea*, *Sclerotinia sclerotiorum*, *Botrytis cinerea* and *Ustilago maydis* have also been studied at the proteome level (Kav *et al.*, 2007). 2-DE

was exploited for identifying the proteins expressed in the formation of the appressorium of *M. grisea* (Kim *et al.*, 2004). Both mycelial proteome and secretome of *S. sclerotiorum* (causal agent of stem rot disease of canola) have been analyzed using 2-DE and ESI-tandem MS (Yajima and Kav, 2006). The mycelial proteome of different strains of *B. cinerea* was compared using 2-DE for the identification of virulence factors (Fernandez-Acero *et.al.*, 2006; Fernandez-Acero *et al.*, 2007).

The above-mentioned reports provide examples where proteomics has been used to gain better understanding of plant-pathogen interactions. In this current study, proteomics based approaches were applied to have more comprehensive knowledge of the molecular events mediating plant-pathogen interactions and those supporting host plant responses and resistance. Although much information about the genes involved in plant diseases is available, not much is reported with respect to proteome modifications associated in plant-pathogen interactions. The biological information gathered from proteomics-based approaches will greatly enhance our understanding about the molecular basis of their interaction. This knowledge will have a clear impact on the future of controlling plant diseases.

1.6. Objectives of this study

The overall goal of the current research was to understand the interaction of canola (*B. napus)* with its two devastating disease causing fungi *L. maculans* and *A. brassicae* and to identify potential strategies to engineer durable disease resistance in canola. In order to achieve the above-mentioned goals, the main objectives of this study are as follows:

1. To discover novel disease tolerance-related proteins of *B. napus* lines developed through conventional breeding between *B. napus* and *B. carinata* upon *A. brassicae* infection using proteomics-based approaches (2-DE and MS/MS) and selected proteins were validated using quantitative real time RT-PCR (q-RT-PCR). One of the selected gene (i.e. germin-like protein) was constitutively over-expressed to generate *Arabidopsis thaliana* transgenics using *Agrobacterium*-mediated transformation and transgenics were evaluated for *A. brassicae* infection.
2. A comparative proteome-level analysis of two isolates of the fungal phytopathogen *Alternaria brassicae*, differing in their virulence.
3. To analyze proteome-level changes in blackleg-susceptible *B. napus* and blackleg-resistant *B. carinata* upon *L. maculans* infection using proteomics-based approaches (2-DE and MS/MS) and selected proteins were validated using q-RT-PCR and western blot analysis.
4. Role of cytokinin in the growth inhibition of fungal pathogens *L. maculans* and *A. brassicae* in both detached leaf and whole plant experiments.

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2. Proteome-level changes in two *Brassica napus* lines exhibiting differential responses to the fungal pathogen *Alternaria brassicae*

2.1. Introduction

Canola (*Brassica napus*) is a major crop cultivated in Western Canada and among the various factors limiting canola productivity is the disease Alternaria blackspot, caused by the fungal pathogen *Alternaria brassicae*. This pathogen causes one of the most destructive diseases affecting *B. napus*, *B. rapa* and *B. juncea* and outbreaks of Alternaria blackspot has resulted in yield reductions of up to 36% (Verma and Saharan, 1994; Saharan, 1993; Rude *et al.*, 1994; Duczek *et al.*, 1998). *A. brassicae* can infect virtually any part of the plant and visible symptoms of infection include chlorotic and necrotic lesions on the leaf, petiole, stem, inflorescence, silique and seed (Verma and Saharan, 1994). Generally, yield reductions as a result of *A. brassicae* infection are the result of infected siliques which lead to premature shattering of pods (Bains and Tewari, 1987). Despite the destructive nature of this pathogen, very little is known about the molecular determinants produced by *A. brassicae* except that it produces an array of phytotoxins including the well-characterized, host-selective toxin destruxin B (Pedras *et al.*, 2002). Although resistance to *A. brassicae* has not been reported in any *Brassica* species, a high degree of tolerance to this pathogen has been reported in *Sinapis alba*, *Camelina sativa*, *Capsella bursa-pastoris* and *Eruca sativa* (Conn *et al.*, 1988; Conn and Tewari, 1986). In many of these cases, resistance is mediated by the production of phytoalexins, for example, the phytoalexin camelexin

has been reported to have antifungal activity against *A. brassicae* and appeared to inhibit the production of destruxin B by *A. brassicae* (Pedras *et al.*, 1998). Additionally, detoxification by plants of the phytotoxins produced by the fungus may also be crucial for mediating resistance to the pathogen, as previously observed in the case of *S. alba* where the toxin destruxin B is detoxified via hydroxylation and glycosylation (Pedras *et al.*, 2001; Pedras *et al.*, 2003). Although extensive information on the phytotoxins produced by *A. brassicae* and the resistance in some plants mediated by the detoxification of the phytotoxins or by the production of phytoalexins is available, to our knowledge, the molecular details accompanying pathogen infection has never been investigated in any host challenged with this pathogen.

Investigating the changes in the transcriptome and proteome that accompany a stress, such as pathogen challenge, can provide valuable information on the specific molecular responses that are occurring in response to the stress. In the post-genomic era, proteome analysis provides real insights into the molecular processes associated with environmental or developmental signals (Gygi and Aebersold, 2000). In fact, proteome analysis techniques including two-dimensional electrophoresis have been used extensively to investigate plant responses to various stresses including pathogen challenge (Subramaniam *et al.*, 2005; Yajima *et al.*, 2004; Zhou *et al.*, 2005; Rampitsch *et al.*, 2006). Based on the proteome-level information obtained through these studies it may be possible to rationally select gene(s) that may have utility in improving plant productivity under conditions of stress (Srivastava *et al.*, 2004; Srivastava *et al.*, 2006). As mentioned previously, resistance to *A. brassicae* has not

been observed in any *Brassica* species. However, *B. carinata*, an amphidiploid member of the cruciferous family, exhibits a higher degree of tolerance to *A. brassicae* (Bansal *et al.*, 1990) and other diseases such as blackleg caused by *Leptosphaeria maculans* which affect canola crops (Sacristan and Gerdemann, 1986; Rimmer and van den Berg, 1992). Using *B. carinata* as a source of resistance, the University of Alberta canola breeding program has developed lines that exhibit increased resistance to blackleg (Subramaniam *et al.*, 2005), whereas the tolerance of these lines to *A. brassicae* has not been previously reported. We have now challenged the blackleg-resistant and -susceptible lines derived from the *B. napus-B. carinata* cross with *A. brassicae* and observed that the two lines differed in the degree of susceptibility/tolerance to *A. brassicae*. In addition, we have characterized the proteome-level changes in these two lines in response to *A. brassicae* and identified a number of proteins whose levels were significantly affected. Moreover, the expression of genes encoding three selected proteins, which were observed to be affected by the pathogen, was investigated at various time points following *A. brassicae*-challenge using quantitative real-time PCR (q-RT-PCR). Our results provide some insights into the molecular basis underlying the differential responses of the two lines to this economically important plant pathogen and may be instrumental in the rational engineering of *B. napus* with increased tolerance.

2.2. Materials and Methods

2.2.1. Plant and fungal material

Blackleg-resistant (02-17044-9) and -susceptible (02- 17034-12) *B. napus* lines derived from an interspecific cross between *B. napus* and *B. carinata* and subsequent backcrosses with *B. napus* (BC2F6) have been previously described (Subramaniam *et al.*, 2005). In this study, seeds from a doubled-haploid derivative of the blackleg-resistant line (03-11116; referred to as line 1) and from the blackleg-susceptible line (02-17034-12 referred to as line 2) were used to evaluate their susceptibility/tolerance to *A. brassicae*. An isolate of *A. brassicae* (UAMH 7476) was kindly provided by Dr. J. P. Tewari, Department of Agricultural, Food and Nutritional Science at the University of Alberta and was used as a source of inoculum.

2.2.2. Plant inoculation

Seeds from both lines were germinated and grown in plastic inserts (7.5 cm x 5 cm; 2 seeds per insert) containing Metro Mix® 290 (Grace Horticultural Products, Ajax, Ontario, Canada) consisting of vermiculite and peat moss. Plants were grown in the greenhouse (22 °C day/18 °C night; 16 h photoperiod) for 2 weeks, fertilized once in 2 weeks with 200 ppm Peters® 20N-20P-20K (Nitrogen-Phosphorus-Potassium). The spore suspension of the pathogen was prepared by scraping mycelia and spores from plates of actively growing fungal cultures into water and filtering the suspension through four layers of cheesecloth to

remove most of the mycelia. The filtered spore suspension was centrifuged at 2000 x g for 5 min and resuspended in deionized water. This centrifugation was repeated one more time in order to ensure a clear spore preparation free of metabolites. After the final wash, supernatant was discarded and spores were resuspended in water containing 0.05% Tween-20. The spores in this suspension were counted using a haemocytometer and the concentration was adjusted to 4 x 10^5 spores/mL. This suspension was used to inoculate 2-week-old seedlings, which had been placed in a humidity chamber (100% RH) for 24 h. Inoculation was performed by gently wounding the true leaves (2 leaves/plant, 2 sites on either side of the midrib) with a pipette tip and placing 25 µL of spore suspension. For the uninoculated controls, 25 µL of sterile deionized water was used (Subramaniam *et al.*, 2005). The plants were returned to the humidity chamber for 24 h after which they were placed in the greenhouse.

2.2.3. Disease evaluation

Disease evaluation was carried out 10 days post-inoculation on a total of 54 plants/treatment in three independent biological replicates (with at least 12 plants in each replicate). The numbers of diseased as well as tolerant plants were recorded and the percentage of disease incidence was calculated. For both lines, disease severity was assessed using a scale described in Buchwaldt and Green (1992) where a score of 0 indicates no symptoms; 1, symptoms detectable with light microscopy only; 2, small chlorotic areas and/or a few small necrotic spots; 3, larger chlorotic areas and/or larger

necrotic spots; 4, severe chlorotic areas and/or many necrotic spots often coalescing; 5, larger areas with chlorosis and necrosis and often wilting of the tissue.

2.2.4. Harvest of plants and protein extraction for two-dimensional gel electrophoresis

Leaves (inoculated and newly formed) were collected from 10 plants for each treatment (12, 24, 48, 72 h post-inoculation) for proteome analysis, flash-frozen in liquid nitrogen and stored at -80 °C until use. Pooled leaf tissues from 10 plants (12, 24, 48 and 72 h post-inoculation with *A. brassicae* and control plants) were ground to a fine powder using liquid nitrogen. Tissue powder (300 mg) was homogenized in 1 mL of acetone containing 10% (w/v) trichloroacetic acid (TCA) and 0.07% dithiothreitol (DTT). This extract was incubated at 20 °C for 1 h, and centrifuged at 15,000 x g for 15 min at 4 °C, and the pellet was washed by resuspension in 1 mL of ice-cold acetone containing 0.07% DTT and centrifuged as described above. The pellet was washed four more times with ice-cold acetone containing 0.07% DTT, air-dried for 20 min and resuspended in 500 µL rehydration/sample buffer (Bio-Rad, Canada) containing 0.1% tributylphosphine (TBP) and incubated overnight at 4 °C. The extracts were vortexed vigorously and centrifuged as above and the supernatants transferred to fresh tubes. The concentration of the protein in the supernatant was determined using a modified Bradford assay (Bio-Rad) and all samples were stored at -20 °C until two-dimensional gel electrophoresis. Extracts from pooled leaf tissue were prepared from at least three independent inoculation experiments.

2.2.5. Two-dimensional electrophoresis

Isoelectric focusing (IEF) of protein extracts in the first dimension as well as separation by SDS-PAGE in the second dimension was performed as previously described (Subramaniam *et al.*, 2005). Briefly, 300 µg of protein in 300 µL of rehydration/sample buffer (8 M urea, 2% CHAPS, 40 mM DTT, 0.2% Bio-Lyte and 2 mM TBP) was used to passively hydrate 17 cm IPG strips (pI 4-7 linear; Bio-Rad). Isoelectric focusing was performed using a Bio-Rad PROTEAN IEF unit programmed to provide an optimum, maximum field strength of 600 V/cm and a 50 µA limit/IPG strip at 10,000 V for 60,000 V h. Prior to the focusing step, the strips were held at 250 V for 15 min to remove charged contaminants and at 500 V for 3 h after focusing to eliminate artifacts due to over- and under-focusing. Prior to second dimension SDS-PAGE, to solubilize focused proteins and allow SDS binding, the focused IPG strips were equilibrated in buffers containing 2% SDS and 130mM DTT for the reduction of sulfhydryl groups followed by a second incubation in buffer containing 135mM iodoacetamide, which alkylates the reduced sulfhydryl groups. Second dimension electrophoresis was carried out on 13% polyacrylamide gels (20 cm x 20 cm, 1 mm thickness) using a PROTEAN II xi system (Bio-Rad) at 45 V/gel until 2 h after the dye front reached the bottom of gel. Gels were stained with silver using the Silver Stain Plus kit (Bio-Rad) and images of the stained gels were acquired using the GS-800 calibrated densitometer (Bio-Rad). For each time-point, images from at least three gels obtained from three independent inoculation experiments were compared.

2.2.6. Image analysis

Two-dimensional gels from the protein extracts of control and inoculated leaf tissue were analyzed using the PDQuest software (Bio-Rad). Gels from three independent biological replicates were used to make the match-sets and individual spots were matched (added or deleted) using software tools. Each set of gels from three replicates were analyzed simultaneously using the Student's *t*-test feature of this software in order to identify protein spots indicating statistically significant differences in levels as a result of pathogen challenge. Spot intensities of control and inoculated gels at different time points were determined using the spot quantification tool and the fold changes from controls were calculated. These spots were excised from the gels using a sterile scalpel and the proteins were identified using ESI-Q-ToFMS/ MS (Subramaniam *et al.*, 2005).

2.2.7. ESI-Q-ToF-MS/MS

Briefly, ESI-Q-ToF-MS/MS analysis for an excised protein sample was performed at the Institute for Biomolecular Design (IBD), University of Alberta, Canada. Processing of the gel pieces was performed in a fully automated fashion on a Mass Prep Station (Micromass, UK). The gel pieces were de-stained, reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide and digested with 6 ng/μL trypsin (Promega Sequencing Grade Modified) in 50 mM ammonium bicarbonate (25 μL) for

5 h at 37 °C. The tryptic peptides were extracted after an enzymatic digestion and subjected to LC/MS/MS analysis on a Micromass Q-ToF-2 mass spectrometer (Micromass) coupled to a Waters CapLC capillary HPLC (Waters Corp., USA). This procedure involved separation on a PicoFrit 5 capillary reversed-phase column (5 µm BioBasic C18, 300 Å pore size, 75 µm ID x 10 cm, 15 µm tip, New Objectives, USA), using a linear water/acetonitrile gradient (0.2% formic acid), with a 300 µm x 5 mm PepMap C18 column (LC Packings, USA) being used as a loading/desalting column. The eluent was introduced directly to the mass spectrometer by electrospray ionization at the tip of the capillary column. Data dependent MS/MS acquisition was performed on detected peptides with a charge state of 2 or 3. Proteins were identified from the MS/MS data through a search of the NCBI non-redundant database using Mascot Daemon (Matrix Science, UK).

2.2.8. q-RT-PCR

Primers for q-RT-PCR were designed using the Primer Premier 3 software (Applied Biosystems Inc., USA) to generate PCR products of approximately 70-80 bp and are shown in Table 2-1. Total RNA was extracted from both plant lines using the QIAGEN RNeasy Plant Mini Kit (Qiagen, Canada) from pooled, leaf tissue obtained from control and pathogen-challenged plants at various time points after infection and treated with RNase-free DNase (Qiagen). First strand cDNA was synthesized by reverse transcription of total RNA (50 ng) using the iScript cDNA synthesis kit (Bio-Rad, USA) and the subsequent PCR reaction was performed with 2 µL of 5 x diluted

cDNA as template, 22.5 pmol of the primers, 5 pmol of the probe and 1 x TaqMan PCR MasterMix (Roche, USA) in a total volume of 20 µL. PCR analysis was performed using an ABI prism 7700 Sequence detector (Applied Biosystems) using the SNP RT template program and comparative relative expression of the various genes was determined using the delta–delta method employing the formula: relative expression = $2^{-[\Delta Ct\ sample\ -\ \Delta Ct\ control]}$ (Livak and Schmittgen, 2001) where Ct refers to the threshold cycle, sample indicates the gene of interest and control indicates the endogenous house-keeping gene. Level of expression in the uninoculated plants was equated to 1 and the relative expression in the inoculated plants was normalized against those levels. The reactions were performed in duplicate and the experiments were repeated at least three times and the results analyzed using the Student's *t*-test.

Table 2-1 Primer sequences for quantitative real-time PCR analysis

Genes	Accession#	Primer pairs and probe used in real time PCR
Germin-like protein	***U21743***	Forward; 5'- GGATTCATCTCCTCTGCCAACT-3'
		Reverse; 5'- CCATGACCTGTCCCGGTTT-3'
		Probe; 5'- TGTCTACGTGCAGACGC-3'
Peptidyl-prolyl *cis-trans* isomerase	***M55018***	Forward; 5'-CGGCAAATCCGGAAAGC-3'
		Reverse; 5'-GATCACGCGGTGGAAAGC-3'
		Probe; 5'- ACTCCACTACAAGGGC-3'
Auxin-induced protein	***H07824***	Forward; 5'- ACGAGACTTTTGGGAACATGGT-3'
		Reverse; 5'- TTGAGACGAAGACGATCTTTGC-3'
		Probe; 5'- CAGTCCGGTCGTCG-3'
Actin	***AF111812***	Forward; 5'- TGGGTTTGCTGGTGACGAT-3'
		Reverse; 5'- TGCCTAGGACGACCAACAATACT-3'
		Probe; 5'- CTCCCAGGGCTGTGTT-3'

2.3 Results and discussion

2.3.1. Response to *A. brassicae*

The two lines of *B. napus* were challenged with *A. brassicae* as described earlier and the effects of the pathogen on these two lines were evaluated. The appearance of the two *B. napus* lines used in the current study 10 days after challenge with *A. brassicae* is shown in Fig. 2-1. Line 1 exhibited severe chlorosis and necrosis that spread from the spot that had been inoculated with *A. brassicae* spores, and had irregular margins extending towards the periphery of the leaf suggesting that it was quite susceptible to *A. brassicae* (Fig. 2-1A and B). In contrast, the leaves of line 2 exhibited localized chlorosis and necrosis only around the inoculated region which clearly indicated that this line was more tolerant to the pathogen than its sister line (Fig. 2-1C and D). The average disease incidence (number of plant infected/ total number of pathogen-inoculated plants x 100) was calculated as 9.13 $\pm$ 0.50% in the *Alternaria*-tolerant line (line 2) and 90.5 $\pm$ 5.91% in *Alternaria* susceptible line (line 1) 10 days after inoculation with the pathogen. Lesions formed on line 1 were generally rated as "4" (severe chlorotic and/or necrotic spots) whereas lesions on line 2 were generally rated as "2" (smaller chlorotic and/or necrotic spots; Buchwaldt and Green, 1992). These results suggest that line 2 is the more tolerant of the two sister lines to *A. brassicae*.

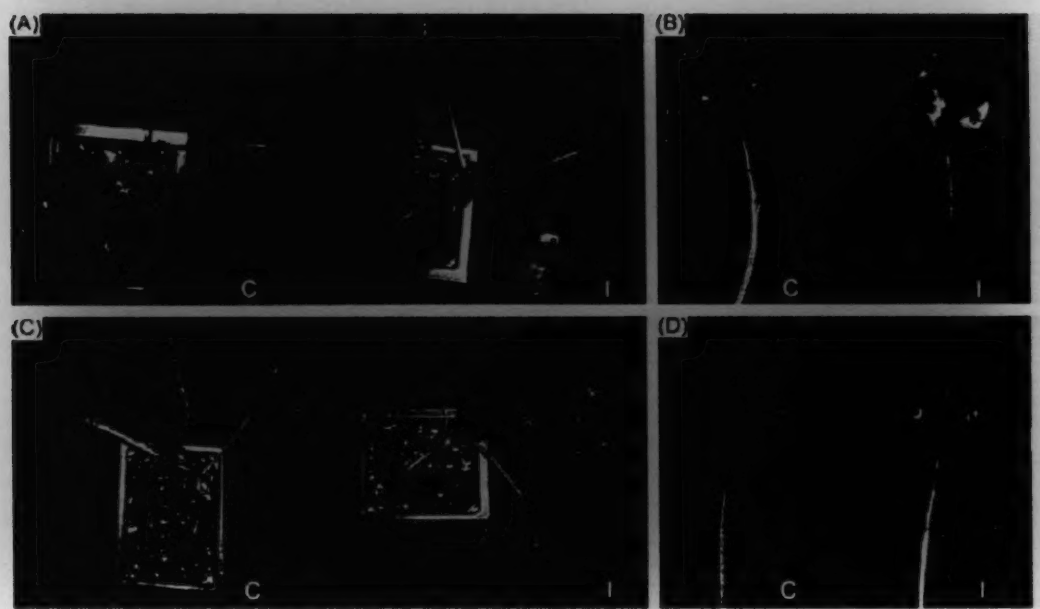

Figure 2-1. Appearance of *B. napus* plants and leaves 10 days after challenge with *A. brassicae*. Panels (A) and (C) show the necrotic lesions induced by the pathogen in the susceptible and tolerant lines, respectively while (B) and (D) are closer views of the challenged leaves from plants shown in (A) and (C). In all the panels the letter 'C' refers to control and 'I' to pathogen challenged plants.

2.3.2. Effect of pathogen on leaf proteomes

The effects of pathogen infection on the leaf proteome of the two *B. napus* lines were investigated at 12, 24, 48 and 72 h after inoculation using two-dimensional electrophoresis and representative images are shown in Figs. 2-2 to 2-11. Three independent gels (one from each of the three biological replicates) were analyzed for each treatment (control plants and pathogen challenged) using the Student's *t*-test and a total of 48 spots were observed to be reproducibly and significantly ($p< 0.05$) altered in intensities in the *Alternaria*-tolerant line (line 2). Representative proteome maps for the uninoculated controls and inoculated plants at various times after pathogen challenge for the *Alternaria*-tolerant line are shown in Figs. 2-2 to 2-5 and the arrows and numbers refer to spots exhibiting significant changes in spot volume after pathogen challenge. Among them, 4 spots were observed at 12 h, 9 at 24 h, 20 at 48 h and 15 at 72 h post-inoculation (Fig. 2-6). Notably, the levels of 41 spots were observed to increase in intensity while the others exhibited a decrease (Fig. 2-6).

In the case of the line susceptible to *A. brassicae* (line 1), we observed a total of 23 spots to be altered in intensity (Figs. 2-7 to 2-11) in response to the pathogen, with 3 at 12 h, 7 at 24 h, 11 at 48 h and 2 at 72 h post-inoculation with the pathogen (Fig. 2-11). In this case, the intensity of only 4 spots was observed to be increased whereas 19 spots decreased in intensity in response to the pathogen (Fig. 2-11). All these protein spots were excised from the gels and subjected to tandem mass spectrometry in order to establish their identities.

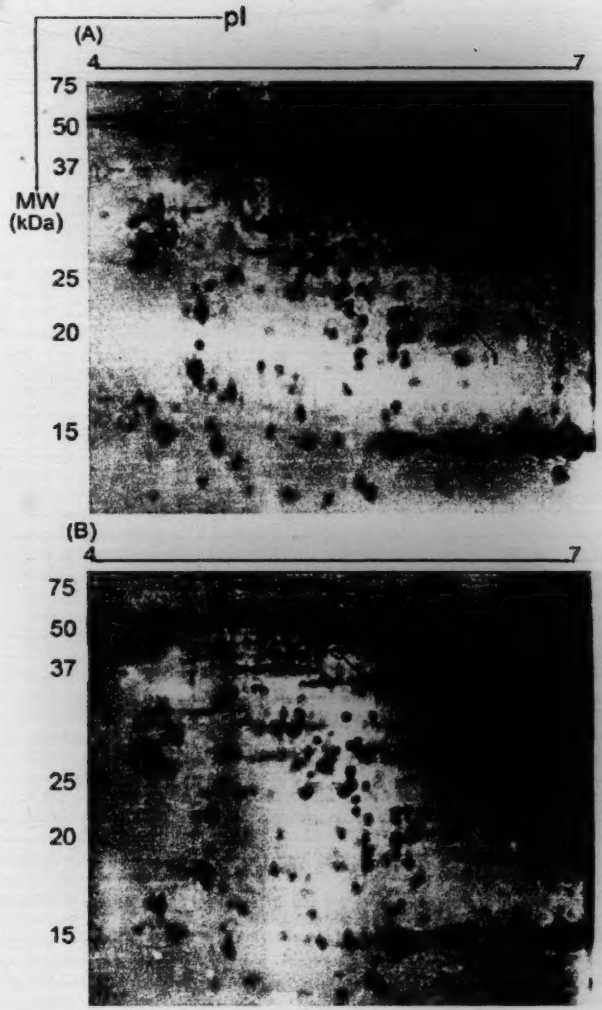

Figure 2-2. Silver-stained images of two-dimensional gels of leaf protein extracted from the tolerant *B. napus* line 12 h after challenge with *A. brassicae*. Gels from control (A) and pathogen-challenged (B) leaf protein extracts are shown where arrows and numbers represent those protein spots whose intensities showed significant changes upon pathogen challenge.

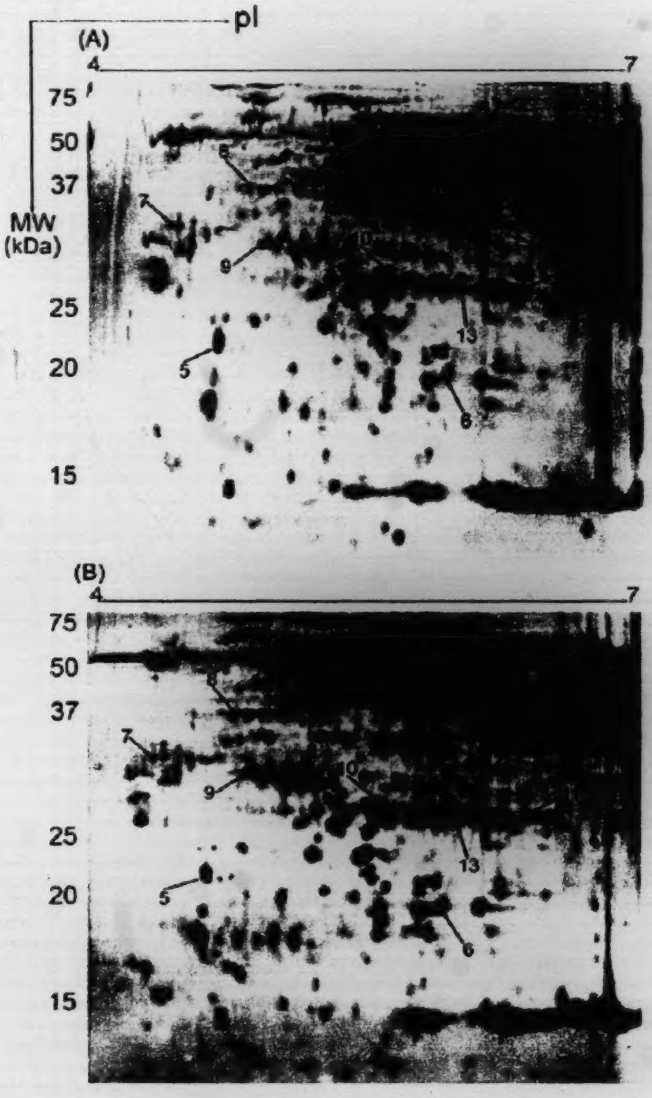

Figure 2-3. Silver-stained images of two-dimensional gels of leaf protein extracted from the tolerant *B. napus* line 24 h after challenge with *A. brassicae*. Gels from control (A) and pathogen-challenged (B) leaf protein extracts are shown where arrows and numbers represent those protein spots whose intensities showed significant changes upon pathogen challenge.

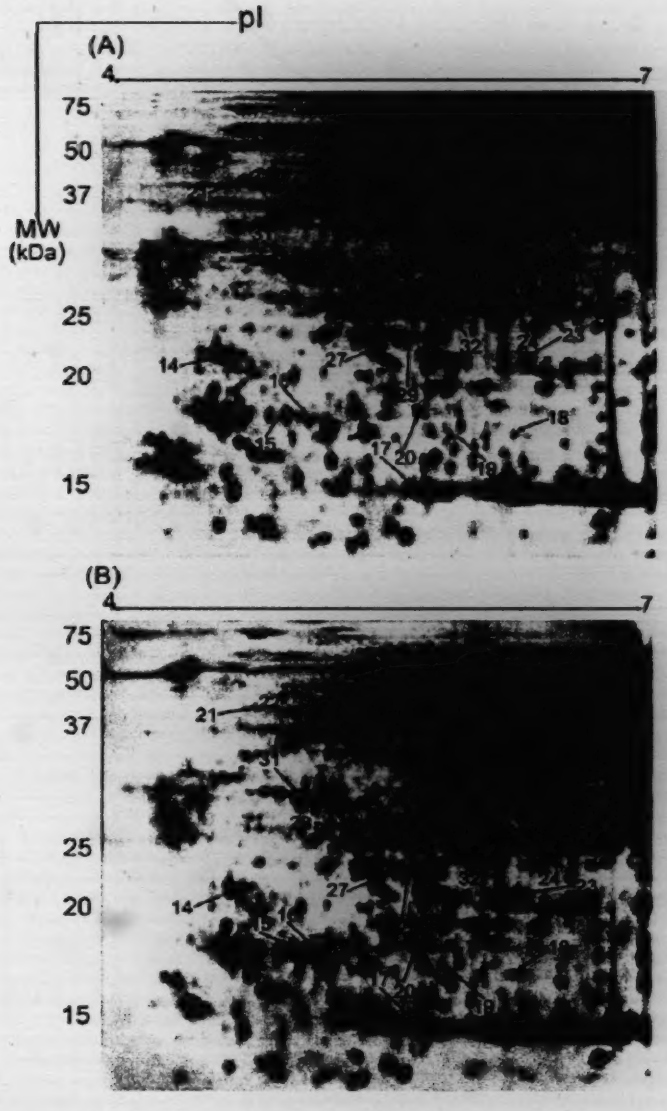

Figure 2-4. Silver-stained images of two-dimensional gels of leaf protein extracted from the tolerant *B. napus* line 48 h after challenge with *A. brassicae*. Gels from control (A) and pathogen-challenged (B) leaf protein extracts are shown where arrows and numbers represent those protein spots whose intensities showed significant changes upon pathogen challenge.

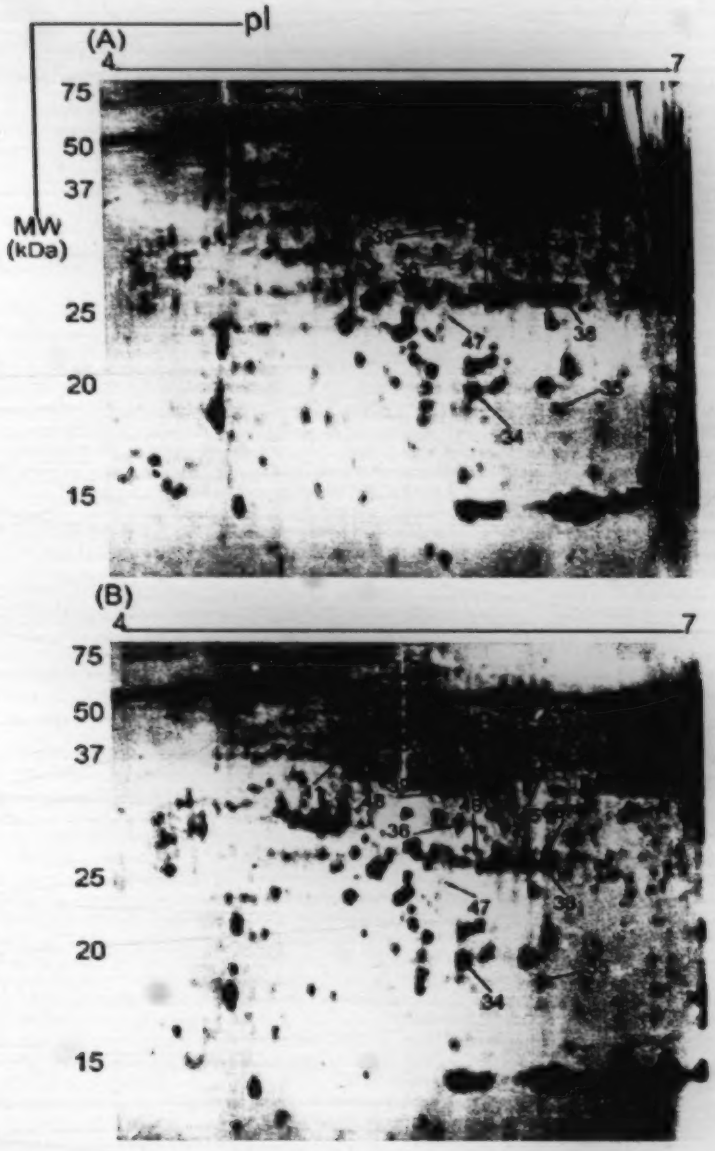

Figure 2-5. Silver-stained images of two-dimensional gels of leaf protein extracted from the tolerant *B. napus* line 72 h after challenge with *A. brassicae*. Gels from control (A) and pathogen-challenged (B) leaf protein extracts are shown where arrows and numbers represent those protein spots whose intensities showed significant changes upon pathogen challenge.

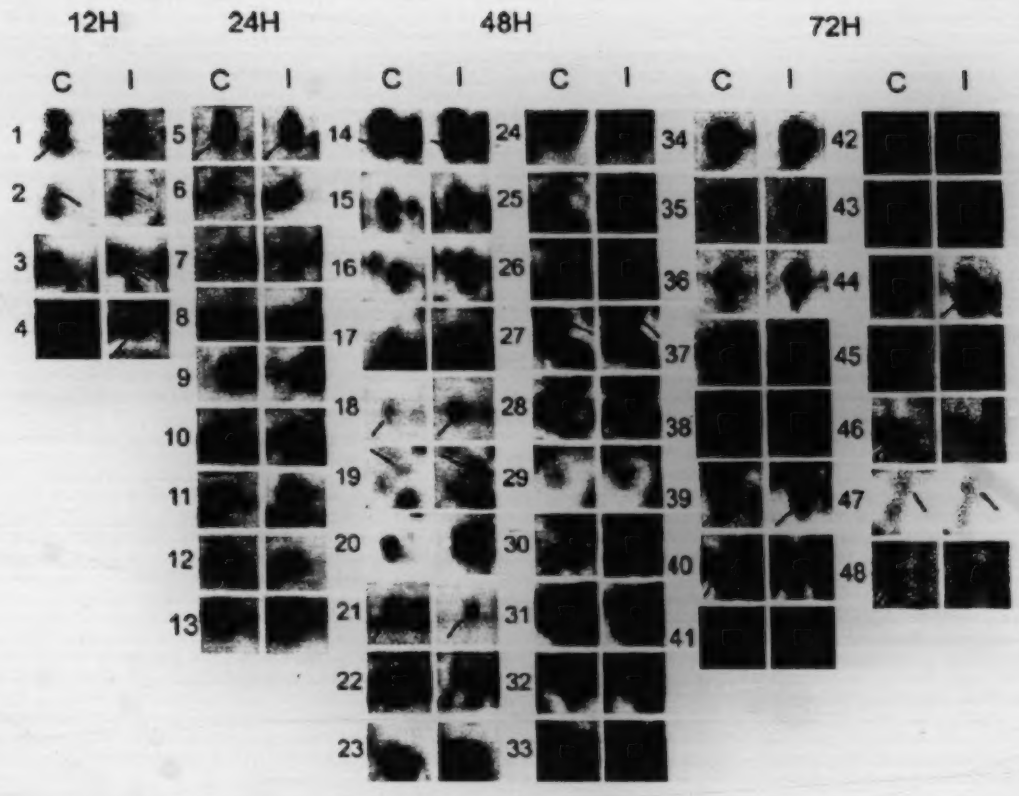

Figure 2-6. A closer view of all the individual spots affected by pathogen challenge at various time points in the *Alternaria*-tolerant line. The spots from control (C) and pathogen challenged (I) are shown.

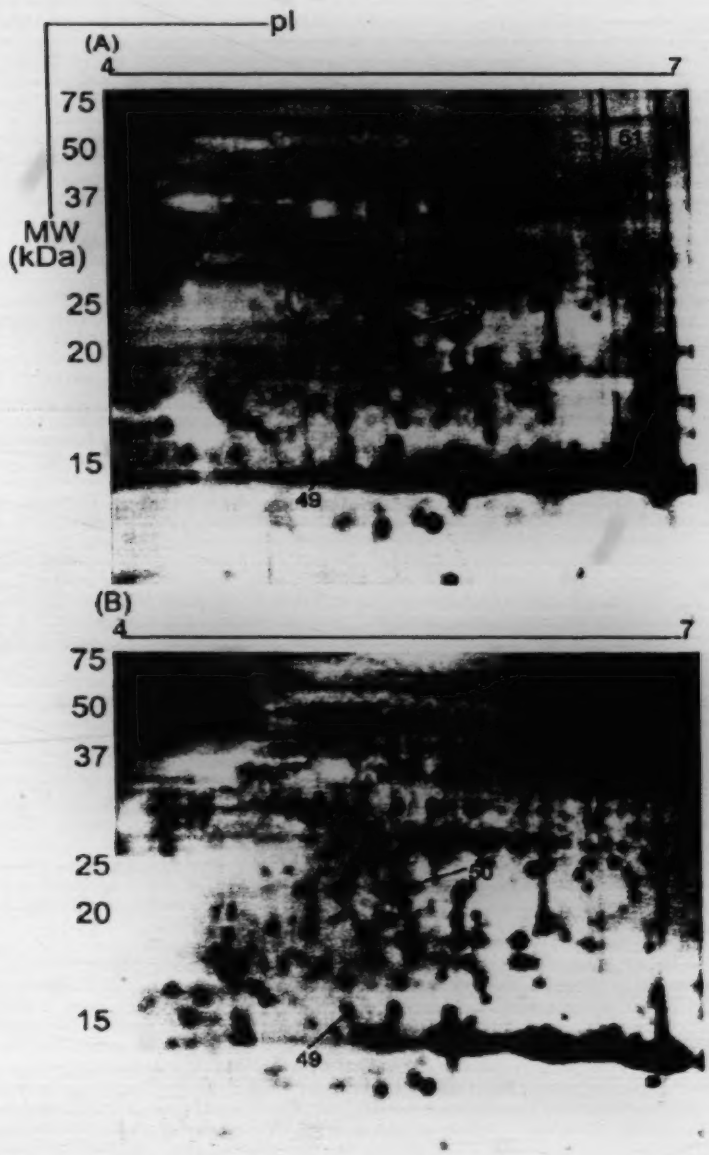

Figure 2-7. Silver-stained images of two-dimensional gels of leaf protein extracted from the susceptible *B. napus* line 12 h after challenge with *A. brassicae*. Gels from control (A) and pathogen-challenged (B) leaf protein extracts are shown where arrows and numbers represent those protein spots whose intensities showed significant changes upon pathogen challenge.

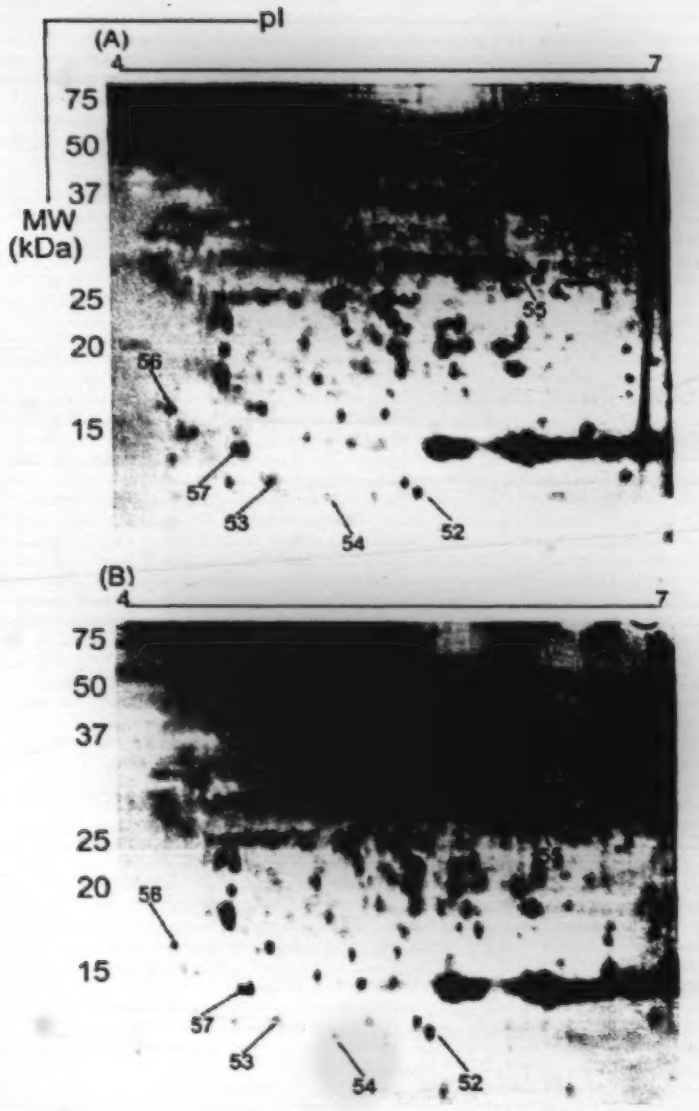

Figure 2-8. Silver-stained images of two-dimensional gels of leaf protein extracted from the susceptible *B. napus* line 24 h after challenge with *A. brassicae*. Gels from control (A) and pathogen-challenged (B) leaf protein extracts are shown where arrows and numbers represent those protein spots whose intensities showed significant changes upon pathogen challenge.

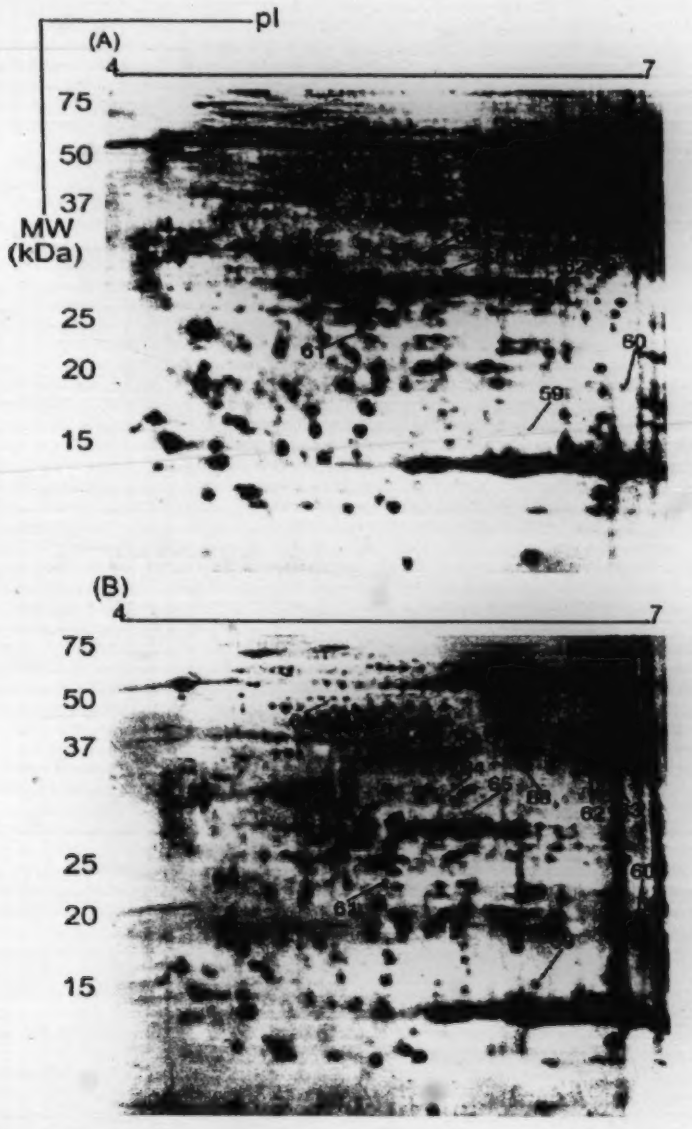

Figure 2-9. Silver-stained images of two-dimensional gels of leaf protein extracted from the susceptible *B. napus* line 48 h after challenge with *A. brassicae*. Gels from control (A) and pathogen-challenged (B) leaf protein extracts are shown where arrows and numbers represent those protein spots whose intensities showed significant changes upon pathogen challenge.

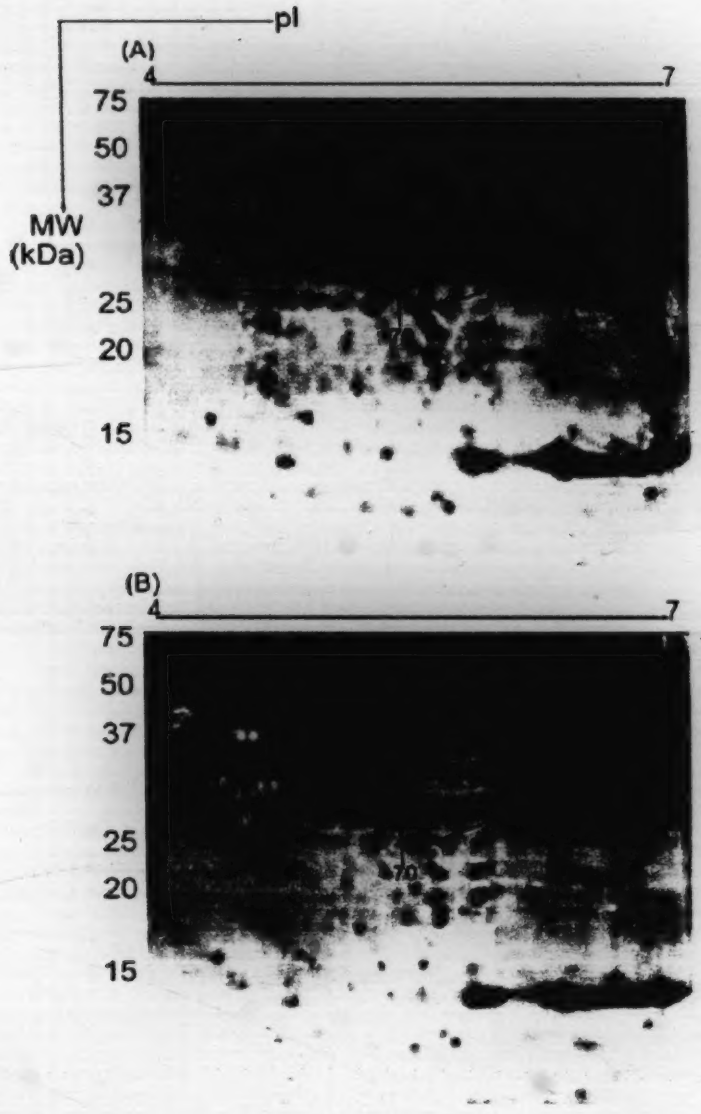

Figure 2-10. Silver-stained images of two-dimensional gels of leaf protein extracted from the susceptible *B. napus* line 72 h after challenge with *A. brassicae*. Gels from control (A) and pathogen-challenged (B) leaf protein extracts are shown where arrows and numbers represent those protein spots whose intensities showed significant changes upon pathogen challenge.

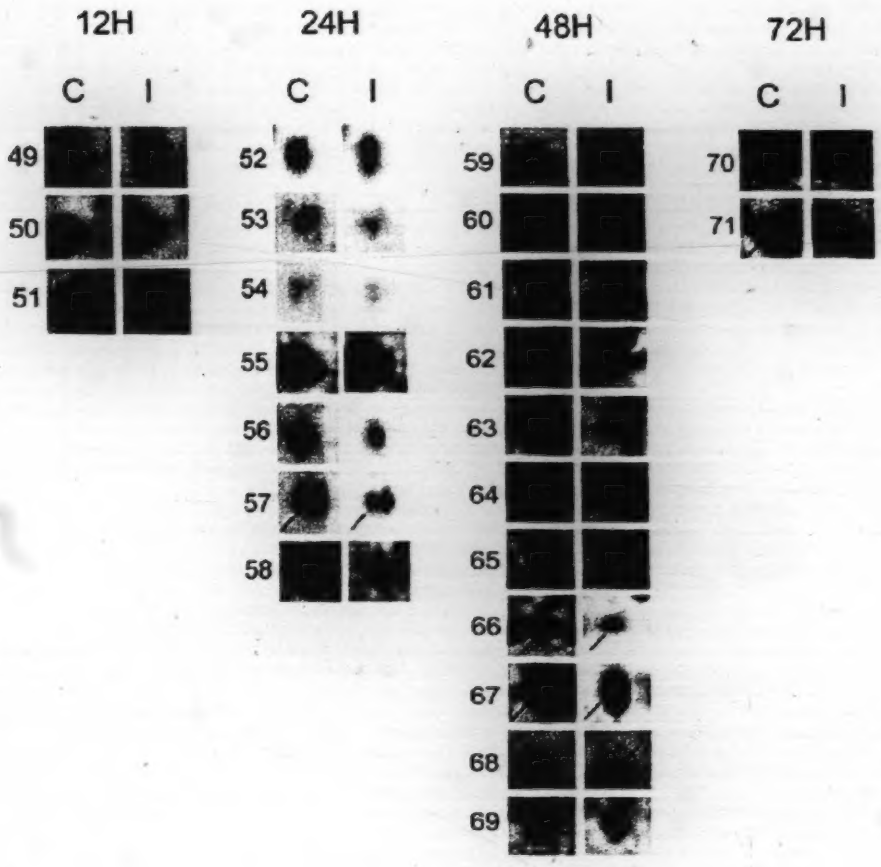

Figure 2-11. A closer view of all the individual spots affected by pathogen challenge at various time points in the *Alternaria*-susceptible line. The spots from control (C) and pathogen challenged (I) are shown.

2.3.3. Identities of differentially expressed proteins upon pathogen infection

The identities of the proteins determined by ESI-Q-ToFMS/ MS, which bases its results on peptide mass fingerprints, are shown in Table 2-2. Using this technique, we were able to identify a total of 38 proteins (out of a total of 71 that were analyzed from the two lines), a success rate of ~53%, which is normal for this type of analysis. Three proteins were identified at 12 h, 6 after 24 h, 11 after 48 h and 10 after 72 h after challenge with *A. brassicae* in the *Alternaria*-tolerant line (line 2) whereas, in the *Alternaria*-susceptible line (line 1) only 1 spot after 12 h, 2 after 24 h, 5 after 48 h and 0 after 72 h of challenge were successfully identified. The identities of the proteins established by MS from these two lines are presented in Table 2 and were ''significant hits'' based on individual peptide ion scores. The ion score is automatically calculated by the Mascot program and is -10 x log(P) where P is the probability that the observed match is a random event. When the ion scores exceed the threshold value for a random event, it indicates sequence identity or extensive homology ($p<0.05$). Normally, the identity of the spot is established as the protein that produced the highest score and, consequently, the best match with its peptide sequence. A number of proteins involved in metabolic pathways, stabilization of protein structure and function, production and detoxification of reactive oxygen species (ROS), and signal transduction were identified as being affected in the tolerant line as a result of pathogen challenge (Table 2-2). Among them, those involved in the production and detoxification of ROS and signal transduction as well as specific metabolic pathways may have a direct bearing on the responses of the plant to the pathogen and are

discussed in greater detail. In the case of the susceptible line, most of the proteins that were identified are involved in primary metabolic pathways with some exceptions (Table 2-2).

Table 2-2 Details of proteins identified from *A. brassicae*-tolerant and -susceptible lines at various times after challenge with the pathogen

Tolerant line:

Spot #	Name of the protein	MS/MS ESI-Q-ToF			[d]Accession. #	[e]Mr/ pI	[f]Status
		[a]PM %	[b]Score	[c]Sequence			
1	Germin-like protein [*Arabidopsis thaliana*]	5 %	61 (>48)	GPQSPSGYSCK	gi|1755184	22022/ 6.81	1.2 ± 0.06 ↑
2	Ribulose 1,5-bisphosphate carboxylase large subunit [*Vigna unguiculata*]	8 %	124 (>49)	LNYYTPEYETK DTDILAAFR DDENVNSQPFMR SQAETGEIK	gi|3114999	52925/ 6.23	1.6 ± 0.12 ↑
3	Plastidic aldolase NPALDP1 [*Nicotiana paniculata*]	7 %	177 (>49)	ALQNTCLK ASSYADELVK YTGEGESEEAK	gi|4827251	42832/ 6.92	2.1 ± 0.31 ↓
6	Peptidylprolyl isomerase ROC4 [*Arabidopsis thaliana*]	10 %	60 (>49)	TLESQETR FEDENFTLK IYACGELPLDA	gi|6899901	28520/ 8.83	1.5 ± 0.23 ↑
7	eEF-1beta [*Arabidopsis thaliana*]	8 %	36 (>49)	SYITGYQASK LVPVGYGIK	gi|398606	25193/ 4.53	1.5 ± 0.00 ↑
8	Sedoheptulose-bisphosphatase precursor [*Arabidopsis thaliana*]	2 %	62 (>49)	ATFDNSEYSK	gi|22136118	42815/ 6.47	1.9 ± 0.29 ↑
9	Inorganic pyrophosphatase-like protein [*Arabidopsis thaliana*]	3 %	64 (>49)	IVAISLDDPK	gi|21592878	33658/ 5.55	1.4 ± 0.00 ↑

11	Triose phosphate isomerase [*Arabidopsis thaliana*]	18%	300 (>48)	VAYALAQGLK EAGSTMDVVAAQTK NVSADVAATTR IIYGGSVNGGNCK	gi|7076787	27380/ 5.24	1.5 ± 0.23 ↑
12	Sedoheptulose-bisphosphatase precursor [*Arabidopsis thaliana*]	2 %	62 (>48)	ATFDNSEYSK	gi|7263568	42815/ 6.47	2.2 ± 0.89 ↑
16	Peroxiredoxin [*Hyacinthus orientalis*]	8 %	63 (>48)	YALLAEDGVVK	gi|42565527	14120/ 5.43	4.4 ± 1.47 ↑
19	Nucleoside diphosphate kinase Ia [*Arabidopsis thaliana*]	13 %	102 (>49)	GDLAVQTGR TDPLQAEPGTIR	gi|3063661	17133/ 5.54	2.5 ± 0.79 ↑
20	Glutathione peroxidase [*Malus x domestica*]	13 %	91 (>48)	GNDVDLSTYK YAPTTSPLSIEK	gi|33308408	18690/ 6.14	1.6 ± 0.06 ↑
25	mRNA binding protein precursor-like [*Arabidopsis thaliana*]	2.5%	89 (>49)	FSEIVSGGGK TNLPEDLKER DCEEWFFDR	gi|7573443	44074/ 8.54	2.0 ± 0.45 ↑
26	Cinnamyl alcohol dehydrogenase [*Brassica rapa*]	24 %	203 (>48)	VTVISSSPSK MVGGSDIGGMK LGADSFLVSSDPQK	gi|6683965	38948/ 6.9	1.6 ± 0.03 ↑
28	Chaperonin 10 [*Arabidopsis thaliana*]	13 %	146 (>49)	DGSNYIALR TAGGLLLTETTK EDDIVGILETEDIK	gi|3057150	26913/ 8.86	1.3 ± 0.15 ↓
29	Chaperonin 10 [*Arabidopsis thaliana*]	4 %	86 (>48)	TAGGLLLTETTK	gi|3057150	26913/ 8.8	3.9 ± 1.68 ↑
30	Chloroplast NAD-MDH [*Arabidopsis thaliana*]	2 %	86 (>48)	IQNAGTEVVDAK	gi|3256066	42623/ 8.48	1.6 ± 0.19 ↑

31	Putative protein 1 photosystem II oxygen-evolving complex [*Arabidopsis thaliana*]	29 %	477 (>49)	NAPPDFQNTK NTAASVGEITLK LTYDEIQSK VPFLFTVK FLVPSYR RLTYDEIQSK FCFEPTSFTVK GDEEELSKENVK QLDASGKPDNFTGK GGSTGYDNAVALPA GGR	gi|4835233	37078/ 6.78	1.4 ± 0.15 ↓
32	Putative dehydroascorbate reductase [*Brassica rapa* subsp. pekinensis]	19 %	109 (>49)	THDGPFIAGGK ALFSLDSFEK	gi|33285914	12029/ 6.15	1.3 ± 0.09 ↑
33	F18O14.33 [*Arabidopsis thaliana*]	2 %	92 (>48)	VSAVDLSLAPK	gi|8778432	50585/ 6.82	1.4 ± 0.12 ↑
34	Peptidylprolyl isomerase ROC4 [*Arabidopsis thaliana*]	10 %	136 (>48)	TLESQETR FEDENFTLK IYACGELPLDA	gi|6899901	28520/ 8.83	1.4 ± 0.13 ↑
36	Carbonic anhydrase [*Lycopersicon esculentum*]	6 %	59 (>48)	YDTNPALYGELAK GGYYDFVK	gi|56562177	34845/ 6.67	2.2 ± 0.44 ↑
37	Triose phosphate isomerase [*Arabidopsis thaliana*]	13 %	173 (>48)	VAYALAQGLK EAGSTMDVVAAQTK NVSADVAATTR	gi|7076787	27380/ 5.24	1.5 ± 0.09 ↑
38	Putative enoyl-CoA hydratase [*Bradyrhizobium japonicum*]	2 %	51 (>48)	EAIAETKR	gi|27381374	33104/ 5.53	3.2 ± 1.09 ↑
39	Cysteine synthase [*Arabidopsis thaliana*]	9 %	129 (>48)	LEIMEPCCSVK LILTMPASMSLER AFGAELVLTEPAK	gi|572517	41976/ 8.13	1.8 ± 0.12 ↑
40	Auxin-induced protein [*Vigna radiate*]	13 %	124 (>49)	VVAAALNPVDAK EGDEVYANVSEK SLGADLAIDYTK GPFPFSR	gi|4056456	32812/ 5.65	1.4 ± 0.03 ↑
44	mRNA binding protein precursor-like [*Arabidopsis thaliana*]	2.5%	108 (>48)	FSEIVSGGGK TVEIVHYDPK TNLPEDLK	gi|7573443	44074/ 8.54	1.5 ± 0.03 ↑

45	Unnamed protein product [*Citrullus lanatus*]	3 %	83 (>48)	TQDGGTEVVEAK	gi|18297	36406/ 8.8	1.3 ± 0.07 ↑
46	Triosephosphate isomerase 1 [*Zea mays*]	5 %	51 (>48)	EAGSTMDVVAAQTK	gi|168647	27236/ 5.52	2.7 ± 1.46 ↑
47	Multicatalytic endopeptidase complex, proteasome precursor, beta subunit [*Arabidopsis thaliana*]	4 %	52 (>48)	TVIINSEGVTR	gi|21592365	24073/ 5.70	2.9 ± 0.74 ↑

Susceptible line:

Spot #	Name of the protein	MS/MS ESI-Q-ToF			[d]Accession. #	[e]Mr/ pI	[f]Status
		[a]PM %	[b]Score	[c]Sequence			
51	mRNA binding protein [*Arabidopsis thaliana*]	8%	132 (>48)	FSEIVSGGGK AVTLDGMAK TNLPEDLK FEEYVK	gi|15229384	44074/ 8.54	2.9 ± 1.0 ↑
55	Germin-like protein [*Arabidopsis thaliana*]	5%	71 (>48)	GPQSPSGYSCK	gi|1755188	22020/ 6.81	3.1 ± 0.48 ↓
58	Putative Malate dehydrogenase [*Arabidopsis thaliana*]	10%	158 (>49)	SQAAALEK ALGQISER LSVPVSDVK MDLTAEELK	gi|21593602	35890/ 6.11	2.1 ± 0.50 ↓
62	Ferredoxin-NADP(+) reductase [*Arabidopsis thaliana*]	4%	181 (>48)	EGQSVGVIADGIDK EQANDKGEK MYIQTR DNTFVYMCGLK	gi|15223753	41484/ 8.51	1.7 ± 0.13 ↓
63	Unnamed protein product [*Citrullus lanatus*]	3%	77 (>48)	TQDGGTEVVEAK	gi|18297	36406/ 8.88	2.1 ± 0.40 ↓

64	Carbonic anhydrase [*Lycopersicon esculentum*]	2%	49 (>48)	VDQITAELK	gi|56562177	34845/ 6.67	2.7 ± 1.40 ↓
67	PGK1 Phosphoglycerate kinase [*Arabidopsis thaliana*]	11%	315 (>48)	SVGDLTSADLK ADLNVPLDDNQTIT DDTR YLIENGAK AHASTEGVTK FAPDANSK	gi|15230595	50195/ 5.91	1.5 ± 0.02 ↓
69	Putative alanine aminotransferase [*Arabidopsis thaliana*]	3%	96 (>48)	FCYNEK LMTDGFNSCK	gi|13430566	53776/ 6.91	1.3 ± 0.07 ↓

[a]Percent sequence coverage.

[b]Mascot score for the most significant hit calculated as described in the Materials and Methods section.

[c]Sequence of the peptide(s) that were matched based on the MS/MS fragmentation patterns.

[d]Accession numbers for the proteins generated by the Mascot search.

[e]Theoretical molecular weight and pI values.

[f]Fold change in the intensity of a spot in the gels from pathogen-challenged tissue over the control gels with arrow direction indicating an increase (↑) or decrease (↓). Average fold changes were calculated from at least three independent gels from three independent biological replicate (i.e. one gel from each replicate). Standard errors of the mean fold changes are also provided.

2.3.4. Proteins involved in metabolic pathways

A number of proteins that were identified in this study as being affected at various times after challenge with the pathogen are those associated with primary metabolic pathways. These include the large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase (Spot #2; Fig. 2-6; Table 2-2), aldolase (Spot #3; Fig. 2-6; Table 2-2), sedoheptulose bisphosphatase (Spots #8 and 12; Fig. 2-6; Table 2-2), triosephosphate isomerase (Spots #11, 37 and 46; Fig. 2-6; Table 2-2), chloroplastic malate dehydrogenase (Spot #30; Fig. 2-6; Table 2-2), putative enoyl-CoA hydratase (Spot #38; Fig. 2-6; Table 2-2) and cysteine synthase (Spot #39; Fig. 2-6; Table 2-2). The intensities of all the above-mentioned enzymes involved in primary metabolic pathways with the exception of aldolase (Spot #3; Fig. 2-6; Table 2-2) were found to be increased in the *Alternaria*-tolerant line (line 2). In addition, an inorganic pyrophosphatase-like protein (Spot #9; Fig. 2-6; Table 2-2) and a protein associated with the oxygen-evolving complex of photosystem II (Spot #31; Fig. 2-6; Table 2-2), were also identified in this line, with the intensity of the former increasing and that of the latter decreasing following pathogen challenge. In the *Alternaria* susceptible line (line 1), as stated earlier, we observed that the intensities of all proteins with the exception of one (Spot #51; Fig. 2-11; Table 2-2) decreased. Because most of the proteins identified in this study were from the tolerant line, we have limited our discussion to those that we believe may play an important role in mediating the observed tolerance to the pathogen.

Our study identified the enzyme carbonic anhydrase (CA; Spot #36; Fig. 2-6; Table 2-2) in the *Alternaria*-tolerant line as being elevated 72 h after challenge with the pathogen and may have a direct role in combating the pathogen. CA has potential involvement in photosynthetic acclimation (Porter and Grodzinsky, 1984) and the requirement for the enzyme varies significantly among photosynthetic organisms. The role of CA in photosynthetic CO_2 fixation is to facilitate the supply of CO_2 to the sites of carboxylation within the chloroplasts. The increase in chloroplastic carbonic anhydrase appeared to be accompanied by a concomitant increase in RUBISCO activity (Majeau and Coleman, 1994). In addition, it has been reported that tobacco chloroplast CA also exhibited salicylic acid (SA)-binding activity (Slaymaker *et al.*, 2002). Furthermore, the silencing of CA gene expression in leaves of *Nicotiana benthamiana* suppressed the *Pto:avr Pto*-mediated hypersensitive reaction (HR) indicating that CA has a role in plant defense (Slaymaker *et al.*, 2002). It is possible that the increase in CA may contribute to the increased tolerance of this *B. napus* line to *A. brassicae*. Furthermore, the intensity of a spot identified as CA (Spot #64; Fig. 2-11; Table 2-2) was observed to decrease in the pathogen-challenged, *Alternaria*-susceptible line further supporting the possible involvement of CA in mediating tolerance to this fungal pathogen.

Another enzyme involved in plant metabolic pathways that may play a role in increasing the tolerance of this line (line 2) to *A. brassicae* is cinnamyl alcohol dehydrogenase (CAD; Spot #26; Fig. 2-6; Table 2-2), which was observed to be increased 48 h after pathogen challenge. CAD catalyzes the final step in phenylpropanoid synthesis specific to the production of lignin monomers (Walter *et*

al., 1988). Besides imparting mechanical rigidity, lignin is resistant to microbial degradation and provides an effective barrier to the invading pathogen. Lignification of infected tissue prevents further colonization of the invading pathogen, in effect isolating the fungus, thereby preventing the spread of the disease. For example, the induction of CAD in bean cell suspension cultures after treatment with fungal elicitor prepared from the mycelial cell wall of the pathogen *Colletotrichum lindemuthianum* has been previously reported (Walter *et al.,* 1988; Grand *et al.,* 1987). Similarly, our proteome-based analysis indicates that enhanced lignin biosynthesis might play a role in mediating the observed effects during this host–pathogen interaction and should be investigated further.

2.3.5. Proteins involved in the production of reactive oxygen species (ROS)

Overproduction of ROS constitutes one of the first responses of plant cells to infection. In addition, ROS can also contribute to programmed cell death (PCD), thereby serving as signal molecules for induction of local and systemic resistance (Corpas *et al.,* 2001). The antioxidant enzymes identified in this study included a germin-like protein in the *Alternaria*-tolerant line (GLP; Spot #1; Fig. 2-6; Table 2-2) observed to be elevated at 12 h after infection. GLPs are functionally diverse proteins that belong to the cupin superfamily and are present in archaea, eubacteria and eukaryota, and are encoded by diverse multigene families in plants (Dunwell *et al.,* 2000). It has been proposed that germins and GLPs represent extracellular superoxide dismutases (SODs), involved in biotic and abiotic stress amelioration (Woo *et al.,*

2000). Furthermore, a SOD isolated from the moss *Barbula unguiculata* has been identified as a GLP (Yamahara *et al.*, 1999). The potential role of GLP in mediating the observed effects is supported by the fact that the expression of several barley germin and GLP genes has been found to be up-regulated during fungal infection of resistant plants (Zhou *et al.*, 1998). Interestingly, our current study also identified a protein whose intensity was reduced in the *Alternaria*-susceptible line 24 h following pathogen challenge as a GLP (Spot #55; Fig. 2-11; Table 2-2), which suggests that the enhanced levels of GLP may have a significant role in mediating the observed responses to the pathogen in these two lines.

2.3.6. Proteins involved in detoxification of ROS

Several proteins involved in detoxifying free radicals generated during stress including pathogen-challenge were identified as being elevated at 48 h after inoculation in the *Alternaria*-tolerant line (line 2). These were peroxiredoxin (Spot #16; Fig. 2-6; Table 2-2), glutathione peroxidase (Spot #20; Fig. 2-6; Table 2-2) and a putative dehydroascorbate reductase (Spot #32; Fig. 2-6; Table 2-2). Components of the ascorbate-glutathione cycle are widely distributed in the cellular compartments where ROS scavenging is required, indicating the importance of this cycle for maintaining the cellular ROS homeostasis. Elevated levels of glutathione peroxidase (GSH-Px) observed in this study may lead to amelioration of infection-induced oxidative stress Dixon *et al.*, 1998). Such a role is further supported by the fact that dehydroascorbate reductase is involved in the detoxification of H_2O_2 via the ascorbate-

glutathione cycle and glutathione-dependent peroxidase (Del Rio *et al.*, 1998) and was identified in this study as being elevated in response to the pathogen. As mentioned earlier, another protein identified with established roles in the detoxification of ROS is a peroxiredoxin (Spot #16; Fig. 2-6; Table 2-2), which also detoxifies H_2O_2 and alkyl hydroperoxides using thioredoxins as electron donors (Poole *et al.*, 2000). Taken together, our observations suggest that the differences in the antioxidant responses, including GLP, peroxiredoxins, dehydroascorbate reductase and glutathione peroxidases may have an important role in mediating plant responses to *A. brassicae* challenge and may play a key role in determining the outcome of the infection process.

2.3.7. ROS signal transduction

Another protein identified in this study as being increased in response to the pathogen in the *Alternaria*-tolerant line(line 2), showing >2-fold increase 48 h after challenge with the pathogen had similarities to nucleoside diphosphate kinase (NDPK) from *Arabidopsis thaliana* (Spot #19; Fig. 2-6; Table 2-2). NDPK is a house-keeping enzyme involved in maintaining cellular levels of all dNTPs (except ATP) and is known to play an important role in signal transduction pathways (Otero, 2000). NDPK has been demonstrated to be involved in mediating abiotic stress as well as hormone responses in plants (Escobar *et al.*, 2001). A direct connection between ROS that accumulate in response to pathogen challenge or abiotic stresses is supported by the observation that the constitutive expression of AtNDPK2 in *A. thaliana* reduced the accumulation of ROS and conferred multiple stress tolerance (Moon *et al.*, 2003).

Furthermore, it was observed that this enhanced tolerance to abiotic stresses resulting from the expression of AtNDPK2 was mediated through the coordination of NDPK2 with MAPK-mediated H_2O_2 signaling. Our current observation that NDPK levels are increased in the tolerant line upon challenge with the pathogen, together with the elevated levels of enzymes detoxifying ROS suggest that ROS-mediated signaling involving NDPK might play an important role in mediating plant responses to *A. brassicae* challenge. A role for NDPK in mediating a response to this pathogen or, in the tolerance to this pathogen has not been previously reported and should be investigated further.

2.3.8. A role for auxin signaling?

Other proteins showing changes upon pathogen challenge in the *Alternaria*-tolerant line (line 2) included peptidylprolyl isomerase (PPIases; Spots #6 and 34; Fig. 2-6; Table 2-2), which showed modest but significant ($p<0.05$) increases 24 and 72 h after pathogen challenge. PPIases, made up of the cyclophilins, the ubiquitous FK506-binding proteins and parvulins, catalyze the cis–trans isomerization of prolyl peptide bonds, a rate limiting step in the folding of newly synthesized proteins (Schmid, 2001). Plant PPIase genes, in particular the cyclophilin genes, have been shown to be induced by a variety of biotic and abiotic stresses suggesting a role in mediating plant responses to stresses (Campo *et al.*, 2004; Godoy *et al.*, 2000; Andreeva *et al.*, 1999). CYP20-3 (previously known as ROC4), identified in this study as showing increased expression at 24 and 72 h, is one of the highly conserved

chloroplast cyclophilins (Lippuner *et al.,* 1994). Within the context of plant–pathogen interaction, elevated levels of PPIases have also been revealed by proteome analysis of germinating maize embryos in response to the pathogen *Fusarium verticilliodes* (Campo *et al.,* 2004). Similarly, Godoy *et al.* (2000) have reported the expression of a potato cyclophilin gene in response to *F. solani* infection. Our current observations suggest that PPIases may play an important role in the *A. brassicae-B. napus* pathosystem as well.

PPIases have also been implicated in auxin-mediated signal transduction (Yajima *et al.,* 2004; Dharmasiri *et al.,* 2003). The interaction of auxin with its cellular receptors triggers a cascade of events leading to the activation of other cellular processes resulting in responses including proton efflux from cells (Macdonald, 1997). One of the effects of auxin is the modification of cell wall components such as lipids and altering the orientation of cell wall polysaccharides (Macdonald, 1997). Some genes involved in mediating cellular response to auxin are known to be involved in the regulation of a ubiquitin-dependent protein degradation process (Leyser, 2002) and belong to the AUX/IAA protein family (Nagpal *et al.,* 2000; Rouse *et al.,* 1998). All the proteins in the AUX/IAA family have two highly conserved proline residues and the conformation (*cis* versus *trans*) of the prolyl peptide bonds are thought to be important for auxin-induced degradation of AUX/IAA proteins via the ubiquitin-dependent pathway (Dharmasiri *et al.,* 2003; Tiwari *et al.,* 2001). Our observation that PPIase levels are increased as a result of challenge with the pathogen in the *B. napus* line suggests a potential role for auxin-mediated signaling during the host-pathogen interaction. Although this suggestion needs to be verified, we identified

another protein spot whose levels were increased at 72 h after pathogen challenge in the *Alternaria*-tolerant line as an auxin-induced protein (Spot #40; Fig. 2-6; Table 2-2). Furthermore, a subunit of the multi-catalytic endopeptidase complex proteasome precursor (Spot #47; Fig. 2-6; Table 2-2) was also observed to be elevated at 72 h in the present study in the *Alternaria* tolerant line. As discussed above, the degradation of auxin responsive proteins via the proteasome degradation pathway is an important part of the auxin signaling process (Dharmasiri and Estelle, 2004) and may be important in mediating tolerance to diseases as well (Gray *et al.*, 2003).

Based on the increased intensities of spots identified as NDPK, PPIase, auxin-responsive protein and the proteasome subunit, it is possible that auxin-mediated ROS signaling may be involved in mediating the higher tolerance of this *B. napus* line to *A. brassicae* perhaps by initiating programmed cell death (PCD) around the point of infection and preventing further spread of the lesion through a hypersensitive reaction. Indeed, such a link between auxin and ROS has been described in a number of studies including one where the cytotoxic effects of indoleacetic acid (IAA) was demonstrated to be the result of increased levels of ROS (De Melo *et al.*, 2004). In addition, the expression of a glutathione transferase/peroxidase gene (GST8) in *A. thaliana* has been shown to be induced by auxin (Bianchi *et al.*, 2002). Furthermore, the application of IAA reduced the disease incidence caused by the tomato pathogen *Fusarium oxysporum lycopersici* and increased plant-growth (Sharaf and Farrag, 2004).

2.3.9. Analysis of gene expression using q-RT-PCR

In order to further investigate whether the observed proteome changes are substantiated by changes in mRNA levels for those proteins involved in ROS-mediated auxin signaling, we investigated the expression of GLP, PPIase and auxin-induced protein. q-RT-PCR analysis was performed using RNA isolated from leaves of unchallenged and *Alternaria*-challenged plants of both lines at 12, 24, 48 and 72 h after challenge. The results obtained are presented in Fig. 2-12 and the differential expression of these three genes in the tolerant line in response to the pathogen is evident. For example, GLP showed a modest increase in protein levels at 12 h after pathogen challenge in the *Alternaria*-tolerant line whereas it showed a significant decrease in intensity of protein at 24 h after pathogen challenge in the *Alternaria* susceptible line. In the q-RT-PCR experiments, we observed a nearly significant ($p < 0.06$) increase in GLP transcript at 24 h and a significant ($p < 0.05$) increase at 48 h after pathogen challenge in the *Alternaria*-tolerant line (Fig. 2-12A). Even though the increase in GLP protein (Table 2-2) as well as transcript levels (Fig. 2-12A) in the *Alternaria*-tolerant line may be modest, the decline of GLP protein level in the *Alternaria*-susceptible line is more dramatic (~3-fold; Table 2-2) but the transcript levels do not show such a dramatic decrease in this line following pathogen challenge (Fig. 2-12A). However, the decrease in GLP protein in the *Alternaria*-susceptible line may be important for the eventual outcome of the host–pathogen interaction, i.e. disease progression.

In the case of PPIase, we observed a significant ($p<0.05$) increase in transcript levels at 12 h after pathogen challenge (Fig. 2-12B) whereas, the increase in protein levels was observed at 24 and 72 h (Table 2-2). Similar to PPIase, an apparent increase in the auxin-induced protein transcript was observed at 48 h (Fig. 2-12C) whereas a significant but modest increase in protein was observed at 72 h (Table 2-2). Our q-RT-PCR results do not correlate well with the observed changes in protein levels for the auxin-induced protein and does not correlate with the specific time points where we observed changes in GLP and PPIase protein levels. However, the results from proteome analysis are probably more meaningful with respect to biological activities of the proteins. This is due to the fact that the presence of more abundant transcripts may not result in increased protein levels owing to post-transcriptional or translational regulation. Also, it is the protein molecules that carry out the biological functions and not the transcripts.

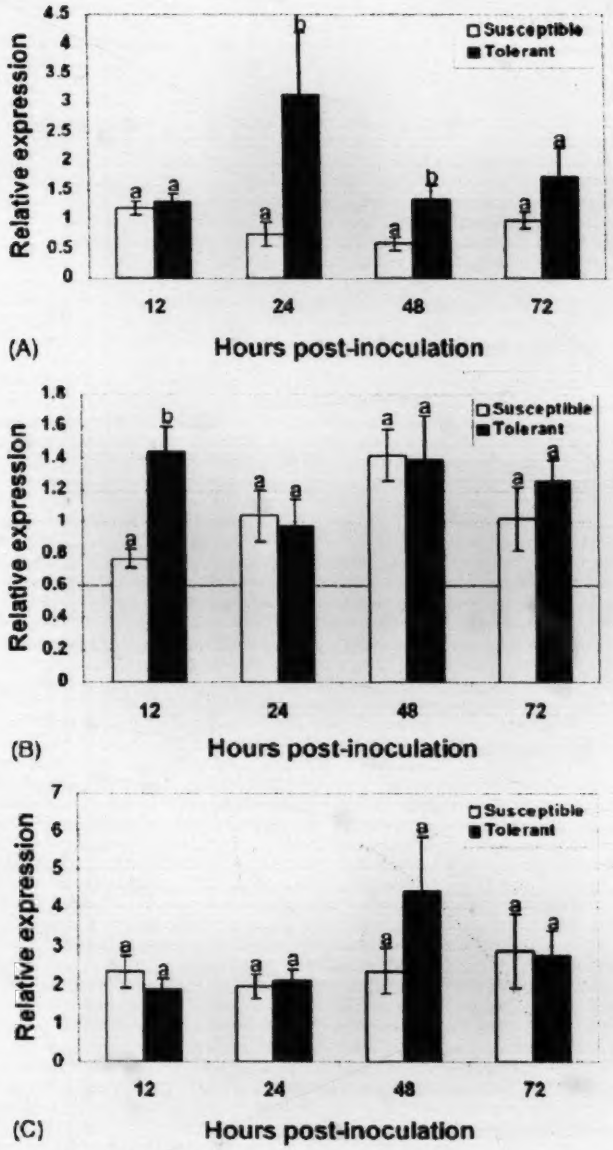

Figure 2-12. q-RT-PCR analysis of gene expression. The relative expression of germin-like protein (A), peptidyl prolyl isomerase (B) and auxin-induced protein (C) at 12, 24, 48 and 72 h following inoculation with *A. brassica* in the susceptible and tolerant lines of *B. napus* are shown. Bars with different letters indicate significant (p<0.05) differences between the treatments.

2.4. Concluding remarks

Proteome analysis has the potential to provide significant insights into the molecular events that occur during plant–pathogen interactions. In this study, we have investigated the proteome-level changes that occur during the *A. brassicae–B.napus* interaction using two-dimensional gel electrophoresis and mass spectrometry. Such an investigation aimed at understanding these events in this pathosystem has not been previously reported. The identities of some of the proteins that showed pathogen-induced changes in quantity in these two lines pointed to specific biochemical processes that may be differentially altered at various time points following pathogen challenge in these two lines. These include enzymes involved in the generation of ROS, their detoxification, signal transduction, as well as responses to the phytohormone auxin. However, the increase in levels of many of these proteins are quite modest, and based on the proteome and q-RT-PCR results reported here it may be premature to propose a definitive role for auxin and ROS signaling in mediating plant responses to this pathogen. Additional investigation into the expression of various genes involved in ROS/auxin signaling pathway at earlier time points after pathogen challenge is warranted and may provide much needed information. Results from such studies may also open novel avenues for engineering durable resistance to this pathogen and are currently underway in our laboratory.

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3. Proteome-level investigation into differences in virulence of two *Alternaria brassicae* isolates

3.1 Introduction

Plant diseases, caused by phytopathogens such as fungi, bacteria, viruses, nematodes and oomycetes are responsible for significant crop losses (Strange and Scott, 2005). Among the pathogens that affect canola (*Brassica napus* L.) in Western Canada and other parts of the world are those that belong to the genus *Alternaria*. Most species of *Alternaria* such as *Alternaria brassicae, A. brassicicola,* and *A. japonica* (formerly *A. raphani*), are responsible for the blackspot disease of crucifers, which can cause significant crop losses, and are transmitted by seeds of many host species belonging to the genera *Brassica* and *Raphanus* (Guillemette *et al.*, 2004a). Most of the commercially available canola varieties are susceptible to this potentially devastating disease (Mondal *et al.,* 2007) and it is important that new varieties be developed to combat the threat of crop losses posed by this pathogen.

A. brassicae can infect canola at all stages of plant growth and any aerial plant part (Verma and Saharan, 1994). Visible symptoms of infection include chlorotic and necrotic lesions on the leaf, petiole, stem, inflorescence, silique and seed (Verma and Saharan, 1994). Infection of siliques can lead to premature pod shatter and shriveled seed, with altered germination efficiency, all of which have been reported in various crucifer hosts such as *Brassica juncea, B. campestris,* and *B. rapa* (Rude *et al.,* 1999; Shrestha *et al.,* 2000). Spores of *A. brassicae* are readily windborne and can be dispersed at great distances throughout the growing season (Guillemette *et al.,* 2004a).

Depending on the virulence of the infecting isolate, outbreaks of blackspot can result in yield reductions ranging from 5-36% (Verma and Saharan, 1994; Rude *et al.*, 1994; Duczek *et al.*, 1998). This fungus produces an array of phytotoxins such as destruxin B and homodestruxin both *in vitro* and *in planta* (Ayer and Pena-Rodriguez, 1987; Bains and Tewari, 1987; Buchwaldt and Jensen, 1991) and these toxic substances function as important virulence factors (Buchwaldt and Green, 1992). However, very little is known about the genes (and proteins) that account for differences in virulence between different isolates of this pathogen. Guillemette *et al.*, (2004b) have reported the identification of two genes, *AbrePsy1* (encoding a non-ribosomal peptide synthetase; NRPS) and *AbreAtr1* (encoding an ATP binding cassette transporter), as putative pathogenicity factors.

In the post-genomic era, proteomics offers a rapid and powerful alternative to genomics-based technology and has been extensively used to characterize and identify novel targets for the design of rational antifungal agents as human/animal therapeutics. For example, comparative proteome-level analysis between two virulent and the avirulent strains of Gram-positive bacterium *Streptococcus suis* type 2 revealed novel proteins that may function as virulence/pathogenicity factors (Zhang and Lu, 2007). Indeed the application of proteomics to investigate fundamental issues in plant biology including the characterization of fungal pathogens and host-pathogen interactions is becoming increasingly popular (Kav *et al.*, 2007). Proteome profiles of several plant pathogens including bacteria, fungi and viruses have been generated in recent years (Kazemi-Pour *et al.*, 2004; Yajima and Kav, 2006; Seifers *et al.*, 2004). Furthermore, proteome analysis has also been recently used as an important tool for identifying new

pathogenicity factors of the fungal plant pathogen *Botrytis cinerea* by comparing mycelial proteomes of two strains differing in virulence (Fernandez-Acero *et al.*, 2006 & 2007) further illustrating an important role played by proteome-based techniques in such investigations into the differences in virulence among plant pathogens.

In this report we describe the proteome-level differences between two isolates of *A. brassicae* that are demonstrated to possess different levels of virulence towards canola. Our results indicate that while, for the most part, the mycelial proteomes of the two isolates are remarkably similar, there are some clear differences in the proteomes that may account for some of the differences in virulence. Our results are discussed within the context of these differences in virulence and may form the basis of novel crop protection strategies in the future.

3.2 Materials and methods

3.2.1 Plant and fungal material

Seedlings from *Brassica napus* (cv. Westar) were grown in plastic inserts (7.5 cm× 5 cm; one seed per insert) filled with Metro Mix® 290 (Grace Horticultural Products, Ajax, Ontario, Canada) in the greenhouse (22 °C day/18 °C night; 16 h photoperiod) for 3 weeks. Plants were fertilized once in 2 weeks with 200 ppm Peters® 20N-20P-20P (Nitrogen-Phosphorus-Potassium). These experiments were repeated at least thrice. Two different isolates of *A. brassicae* (UAMH7476 and

Ontario), used in these experiments were provided by Dr. J. P. Tewari, Department of Agricultural, Food and Nutritional Science, University of Alberta.

3.2.2 Preparation of fungal inoculum and plant inoculation

Fungal isolates of *A. brassicae* UAMH7476 and Ontario were cultured at room temperature (21 ± 2 °C) on V8 juice-rose Bengal agar medium (Degenhardt *et al.*, 1974) in the dark for 12 days. Fungal spore suspensions were prepared in water by scraping spores and mycelia from the plates with a glass-rod. This suspension was filtered through 4-layers of cheesecloth in order to remove mycelia and agar pieces. The suspension was further centrifuged twice at 2000 x g for 5 min in a Sorvall GLC-2 centrifuge (Sorvall; Ontario, Canada) to remove smaller particulate matter, remnants of media and metabolites from the preparation. The washed, pelleted spores were resuspended in water containing 0.05% Tween-20 and counted using a haemocytometer. For inoculation of *B. napus*, 4 x 10^5 spores/mL were prepared for each isolate and 25 µL of spore-suspension was applied on each side of the midrib of wounded leaves. Both detached-leaf and whole plant experiments were performed and repeated as three independent experiments. Disease scoring was carried out using APS Assess software (Image Analysis Software for Plant disease Quantification; American Phytopathological Society) 5 days post-inoculation in case of detached leaves or 10 days after pathogen challenge in whole plant experiments.

3.2.3. Preparation of fungal material for proteome-level analysis

In order to get sufficient mycelial tissue for proteome analysis and also to avoid any other protein contamination in the culture from the media itself, we used PDA (Potato Dextrose Agar; Becton Dickinson, Sparks, MD, USA) media for growth of both *A. brassicae* isolates. Fungal starter cultures were cultured in semisolid PDA media for 15 days in the dark at room temperature and agar containing both mycelia and spores was homogenized in a blender in sterile Milli Q water under aseptic conditions. This blended fungal culture (50 mL) was used to inoculate 250 mL flasks containing 100 mL of liquid PDB (Potato Dextrose Broth; Becton Dickinson, Sparks, MD, USA) media and left stationary in the dark. The mycelial growth on the top layer of the media was removed after 15 days with a sterile spatula and washed 5 times by resuspending in 10 mL of 10 mM Tris HCL (pH 7.8) containing complete EDTA-free protease inhibitor (Roche) and centrifugation at 15,300 g for 10 min at 4 °C. The supernatant was discarded and the washed mycelial tissue was lyophilized. Lyophilized mycelial tissue was ground to a fine powder in liquid nitrogen and homogenized in 10% TCA-acetone and 0.07% dithiothreitol (DTT), vigorously vortexed, incubated at -20 °C for 1 h followed by centrifugation at 21,000 g for 10 min at 4 °C. The supernatant was discarded and the pellet was washed with pre-chilled acetone containing 0.07% DTT thrice by centrifugation as described above. The washed pellet was dried under vacuum to remove residual acetone and the extracted proteins were resolubilized with rehydration/ sample buffer (Bio-Rad) supplemented with 2 mM tributylphosphine (TBP; Bio-Rad). .

3.2.4. Two-dimensional electrophoresis (2-DE)

Mycelial protein preparation (600 µg) was mixed with rehydration/sample buffer (Bio-Rad) to a total volume of 300 µL and loaded on 17 cm IPG strips (pH 3-10, non-linear; Bio-Rad) for passive rehydration overnight. Isoelectric focusing (IEF) was performed using a PROTEAN IEF (Bio-Rad) cell. Focused strips were equilibrated for 20 min in equilibration buffer containing 0.37 M Tris-HCl (pH 8.8) 2% SDS, 20% glycerol, 6 M urea, and 130 mM DTT in order to reduce the proteins. This was followed by a second incubation in the buffer containing 135 mM iodoacetamide (IAA) instead of DTT for an additional 20 min to alkylate the reduced proteins. Following equilibration, SDS-PAGE (13% polyacrylamide gels, 20 cm × 20 cm, 1 mm thickness; containing 0.1% SDS, 0.1% ammonium persulfate, 0.375 M Tris-HCL pH 8.8, 13.3% acrylamide/PDA and 0.04% TEMED) was performed using a PROTEAN II xi electrophoresis cell (Bio-Rad) at 33 V/gel for 22 h 30 min followed by 1 h at 45 V/gel. Two gels were obtained for mycelial sample from each independently cultured fungal mycelia by separating two independently extracted protein samples (3 biological replicates x 2 technical replicates) for a total of 6 gels.

3.2.5. Image analysis

Analysis of the gel images was performed using PDQuest (version 7.3.1) 2-DE image analysis software (Bio-Rad). A match set of 12 gels consisting of 6 gels per isolate of the fungus was created and spots on the gels were matched using the

automated spot detection and matching tools of the software. This was followed by a manual verification of the spots and a Student's *t*-test (using the built in *t*-test feature of the software) in order to identify those spots that were exhibiting reproducible and statistically significant ($p<0.05$) changes in spot intensity between the two isolates of the fungus. This process led to the identification of unique spots and the determination of intensities for significantly altered spots. These spots were selected and excised from the gels for subsequent MS/MS analysis.

3.2.6. Identification of proteins using tandem MS

We performed MS/MS analysis using MALDI TOF/TOF or LC-MS/MS. For MALDI TOF/TOF analysis, in-gel tryptic digests were applied to an AnchorChip plate (Bruker) and analyzed using a Bruker Ultraflex TOF/TOF mass spectrometer (Bruker) as previously described by Zhang *et al.*, (2007). Essentially, the sample (1 µL) was applied to the plate and allowed to dry followed by the application of 0.8 µL of a 0.5 mg/mL CHCA (alpha-cyano-4-hydoxycinnamic acid) in 70% acetonitrile and 0.1% TFA and washed with 0.5 % TFA. Mass spectra and tandem mass spectra were then acquired in an automated fashion using the AutoXecute function. The spectra were then processed using Flexanalysis and mgf files produced using BioTools which were used to search the databases using Mascot (Matrix Sciences, UK, 1999).

In some instances, in gel tryptic digests were analyzed by LC MS/MS using a Bruker 9.4 T Apex-Qe FTICR (Fourier Transform Ion Cyclotron Resonance). LC separations were performed using an Agilent 1100 Capillary HPLC system operated at

10 µL/min onto a 75 µm x 10 cm, 5 µm particle size C18 reversed phase column (Michrom Bioresources). Peptides were eluted using a linear 2% B/min gradient starting at 95% A (where A is 0.2% formic acid in water, B is 0.2% formic acid in acetonitrile). Mass measurements were taken from m/z 350-1600 and data dependent MS/MS was performed on doubly and triply charged precursors. A cell fill time of 0.5 sec was used for MS measurements and 1 sec for MS/MS. Data files were processed using Apex Data Analysis software generating an mgf file that was used to search databases using Mascot. For non-annotated hypothetical and predicted proteins, NCBI's BLASTp was used to assign probable functions to them with conserved domains. Functions of the identified proteins discussed in this study were based on the information available in the literature and also verified using gene ontology in Pfam server (http://pfam.sanger.ac.uk/).

3.2.7. Histological studies

Pathogen-inoculated (both isolates of *A. brassicae*) leaves of *B. napus* cv. Westar as well as mock-inoculated controls were cut into small (5 mm x 10 mm) pieces and fixed in FAA (formalin, acetic acid and ethyl alcohol; Yeung and Saxena, 2005) under vacuum at room temperature overnight. Following fixation, these tissue segments were subsequently dehydrated in a series of graded ethanol/ water solutions, changed to toluene and later infiltrated with Paraplast® using a Fisher Histomatic Tissue Processor (Model 166; Pittsburgh, PA, USA). Paradermal sections (7 µm thickness) were prepared using an AO Rotary microtome (Spencer 820; Buffalo, NY,

USA), affixed to the glass slides, de-paraffinated with toluene, rehydrated in 50% ethanol and stained with Aniline blue in Lacto-phenol for 10 min (Larone, 1995). Stained sections were rinsed with water (3 x 3 min each) and counterstained with acidified Eosin Y for 1 min (Dougherty, 1981), dehydrated in ethanol followed by toluene and mounted with DPX® (Electron Microscopy Sciences, Hatfield, PA, USA) mounting medium. The sections were viewed with a Leica DM *RXA* microscope (Leica Microsystems, Wetzlar, Germany), analyzed using Macrofire™ software (Optronics, Goleta, CA, USA) and photographed with an Optronics digital camera (Optronics, Goleta, CA, USA).

3.2.8. Statistical analysis

Disease severity score results of this study were statistically analyzed using analysis of variance (ANOVA) with the mixed model procedure of SAS version 9.1 (Statistical Analysis system; SAS Institute Inc., Cary, NC, USA).

3.3. Results and Discussion

3.3.1. Effect of *A. brassicae* isolates on *B. napus*

In order to study the phenotypic effects of both isolates of *A. brassicae*, detached leaves and whole plant of highly-susceptible *B. napus* were inoculated with the isolates. In this study, significant differences were observed between the two

isolates of *A. brassicae* in terms of disease severity. Both isolates were able to induce symptoms of blackspot disease but less necrosis and chlorosis was observed in the detached leaves and the whole-plants infected by the UAMH7476 isolate (Figs. 3-1A & 1B) compared to the Ontario isolate (Figs. 3-1A & B). Detached leaves and whole-plants displayed severe symptoms of necrosis and chlorosis starting from the point of inoculation gradually spreading towards the leaf periphery upon infection with the Ontario isolate (Figs. 3-1A & B). This infection process was slower in the case of *A. brassicae* isolate UAMH7476 compared to the other isolate indicating the more virulent nature of the Ontario isolate. Disease severity scores (lesion size/ total leaf area x 100) were calculated on detached leaves as well as whole plants inoculated with both isolates. In the case of detached leaf experiments, disease severity scores for plants inoculated with UAMH7476, and the Ontario isolate were 4.82% ± 0.51 and 11.23% ± 1.47 respectively. Similarly, disease severity scores of 2.88% ± 0.43 for UAMH7476-inoculated and 8.34% ± 1.06 for Ontario-inoculated were observed in whole-plant experiments further confirming the fact that the Ontario isolate was more virulent of the two used in this study.

3.3.2. Histological studies

The interaction of the two isolates of *A. brassicae* with *B. napus* was also investigated using light microscopy. Mock-inoculated controls revealed dead and injured cells at the wounding site in *B. napus* (Fig. 3-2A). Fungal mycelial growth could be observed in *B. napus* plants inoculated with both the less virulent

UAMH7476 (Fig. 3-2B) and the Ontario (Fig. 3-2C) isolates of *A. brassicae*. Plants inoculated with the Ontario isolate (Fig. 3-2C) displayed profuse mycelial growth with faster spread towards the non-inoculated area from the wounding site of the leaves as compared to plants inoculated with UAMH7476 isolate (Fig. 3-2B). Our histological observations correlate well with phenotypic observation of *B. napus*-infected with the two different isolates of fungus and confirm the more virulent nature of the Ontario isolate.

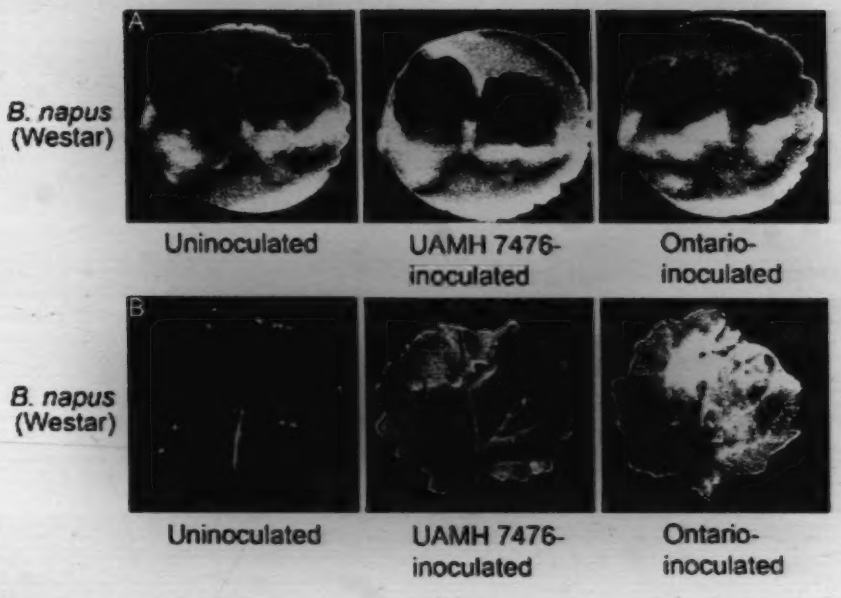

Figure 3-1: Disease symptoms induced by the less-virulent UAMH 7476 and the more-virulent Ontario isolate of *A. brassicae* on detached leaves (A) and whole-plants (B) of *Brassica napus* cv Westar.

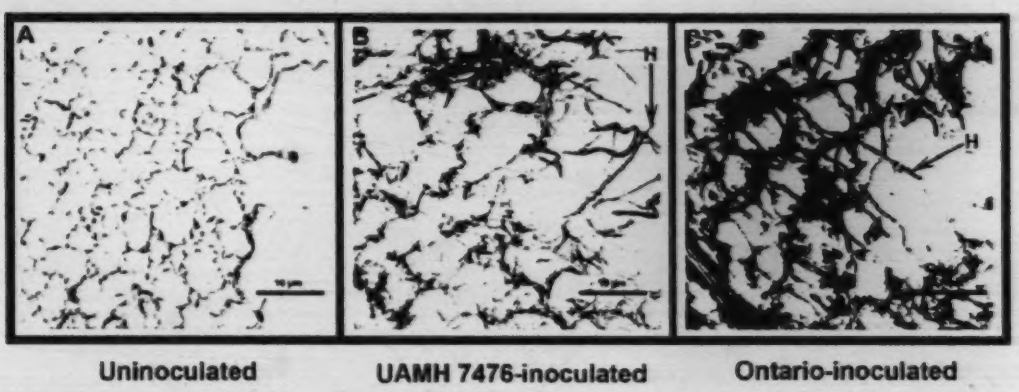

Figure 3-2. Light microscopy images of *B. napus* (A) mock-inoculated control (B) inoculated with less-virulent UAMH7476 isolate and (C) with the more-virulent Ontario isolate. Arrows indicated presence of hypha (H).

3.3.3. Comparison between the whole-proteome of both isolates of *A. brassicae*

For comparative proteome-level analysis, mycelial protein extracts from both isolates of *A. brassicae* were used for 2-DE separation. We then used the automated spot detection feature of the PDQuest software to detect and match spots between gels of both isolates. Addition of missed spots and deletion of artifacts were marked manually on the gels. We used the built-in Student's *t*-test module in PDQuest software at ($p<0.05$) on a matchset, which aided in testing the significant changes in the abundance of a given protein spot in one isolate as compared to the other. A total of ~300 spots were resolved after staining with Coomassie Blue in gels of both isolates in the pH range of 3-10. This study revealed quantitative differences in the expression pattern of proteins between the two isolates of *A. brassicae* and only reproducible changes were considered for further protein identification purposes.

Eighteen spots were detected in the mycelial protein extracts of both isolates as a result of the PDQuest analysis and were of interest to us. Among them, two protein spots were observed to be unique to the protein extracts of the less virulent UAMH7476 (Spots# 1 and 2; Figs. 3-3A & 3-4; Table 3-1) and 5 were unique for the more virulent Ontario isolate (Spots# 7, 8, 9, 10 and 11; Figs. 3-3B & 3-4C; Table 3-1). We also identified protein spots that differed significantly ($p<0.05$) in abundance between the two isolates. Four protein spots with higher intensities in UAMH 7476 were observed (Spots# 3, 4, 5 and 6; Figs. 3-3A & 3-4C; Table 3-1) and seven with higher spot intensities in the Ontario isolate (Spots# 12, 13, 14, 15, 16, 17, and 18;

Figs. 3-3B & 3-4C; Table 3-1) were identified. The identities of these eighteen protein spots were established by tandem MS and are discussed below.

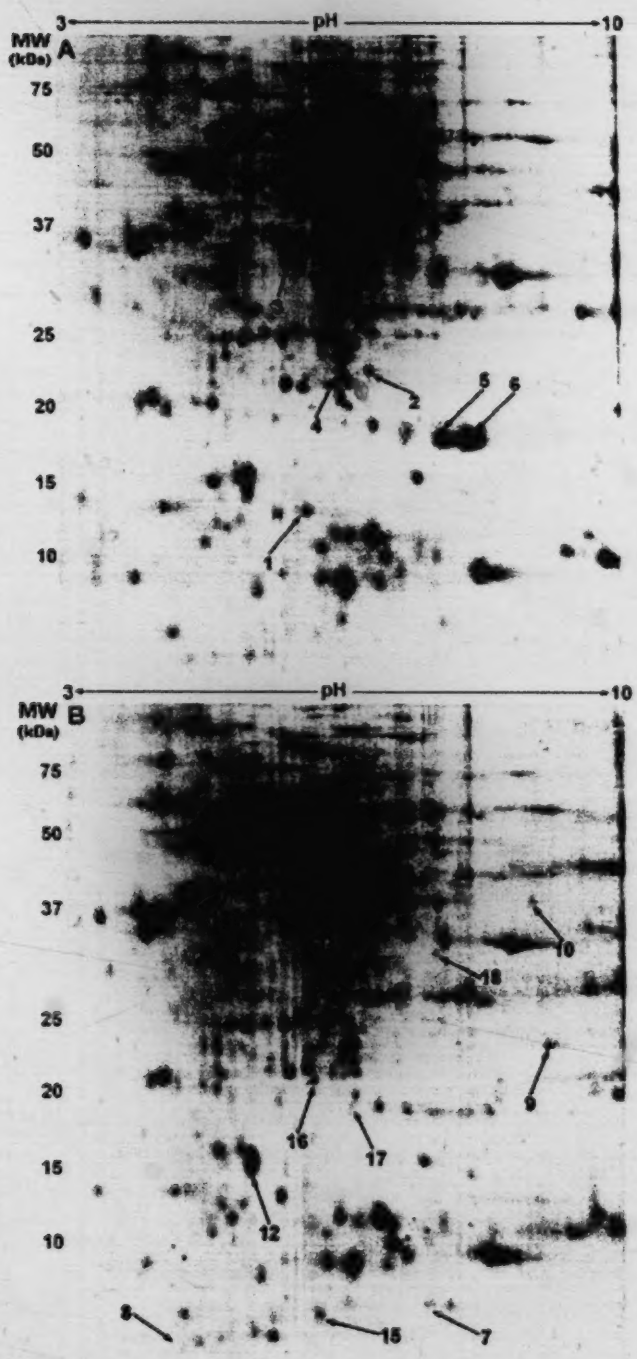

Figure 3-3. Images of two-dimensional gels of mycelial proteomes of a less virulent isolate UAMH7476 (A) and more virulent Ontario isolate (B) of *A. brassicae*.

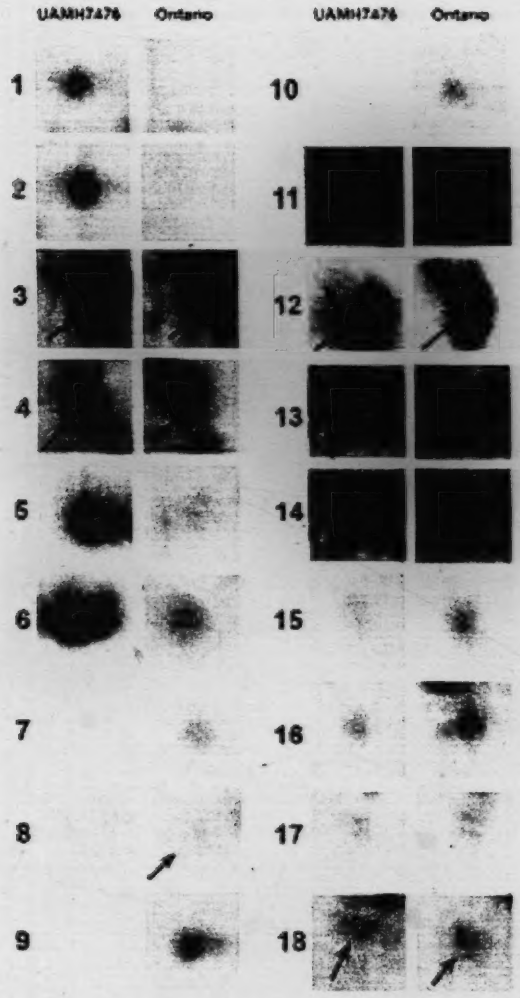

Figure 3-4. Panel shows expanded views of the protein spots observed as unique or with altered abundance in either of the two isolates (UAMH7476 and Ontario).

3.3.4. Identities of proteins

As mentioned above, we detected two unique spots in the gels of the less virulent UAMH7476 isolate and 5 in the gels from the more virulent Ontario isolate. These proteins were identified using MS/MS and database searches and the identities are presented in Table 3-1. However, among the 5 unique spots observed in the more virulent Ontario isolate, only one (Spot# 11; Figs. 3-3B & 3-4C; Table 3-1) and one out of the two in UAMH7476 (Spot# 2; Figs. 3-3A & 3-4C; Table 3-1) generated matches to proteins in the database that was based on more than one peptide.

In addition to the unique proteins, the proteins with altered spot intensities in the two isolates were also identified (Table 3-1). In the case of UAMH7476 isolate, we detected a total of four proteins with altered intensity among which only two generated "hits" with scores at or more than the threshold value. Even those two protein matches were based on a single peptide and are presented in Table 3-1. With respect to the more virulent Ontario isolate, 7 proteins were observed to increase in intensity among which six generated matches that were based on the identification of more than one peptide (Table 3-1). Thus, we were able to generate better MS/MS data with the proteins from the Ontario isolate compared to the less-virulent UAMH7476 isolate.

The majority of proteins identified in isolate UAMH7476 were hypothetical proteins except one (Spot# 4; Figs. 3-3A & 3-4C; Table 3-1) that was identified as a putative proteasome component Prs3. One of the potential limitations of proteome-based investigations into fungal plant pathogens is the lack of complete genome

sequence information. Therefore, most of the proteins identified were hypothetical proteins with unknown functions. As a result, we used NCBI's BLASTp to assign probable functions to hypothetical proteins with conserved domain. NCBI's Conserved Domain Database (CDD) was also considered for presence of highly conserved domains. Our findings are discussed below within the context of a role for these proteins in virulence of this pathogen.

The first hypothetical protein identified in this study was SNOG_09590 with expected value 1e-50 (Spot# 2; Figs. 3-3A & 3-4C; Table 3-1) from the less virulent UAMH7476 isolate. According to the CDD database search, this protein contains a conserved nitroreductase family protein domain and was a unique protein in the extracts of this isolate. The nitroreductase family consists of a group of FMN- or FAD-dependent and NAD(P)H-dependent enzymes, which are able to metabolize nitrosubstituted compounds. In bacterial pathogens such as *Burkholderia pseudomallei* and *B. mallei,* which require iron for optimal growth, the gene encoding a nitroreductase family protein was observed to be highly induced during bacterial growth in iron-restricted conditions (Tuanyok *et al.*, 2005). Also, nitroreductase was observed to be up-regulated under oxygen starvation conditions in *Mycobacterium tuberculosis* (Purkayastha *et al.*, 2002). The unique protein identified in the less virulent isolate might also have a role under nutrient-deficient conditions aiding the fungal growth but nothing has been reported about the role of nitroreductase family protein with respect to virulence of pathogen. As mentioned previously, the remaining proteins detected in this isolate did not generate matches based on more than one peptide and are not considered further in our discussion.

Among the five unique proteins that were detected from the gels of the more virulent Ontario isolate of *A. brassicae*, only SNOG_03354 with expected value 5e-167 (Spot# 11; Figs. 3-3B & 3-4C; Table 3-1) which generated a match based on three peptides was considered further. This protein contains a conserved actin related protein 2/3 complex subunit domain. Actin related protein (Arp) 2/3 complex is important for actin-based motility and reorganization of actin networks during endocytosis (Deeks and Hussey 2005; Martin *et al.*, 2006). Although the precise roles of Arp2/3 in *A. brassicae* and its relevance to virulence is not known at this time, it is tempting to propose an important role for this protein in endocytotic (or other) processes that require actin reorganization in the virulence of this pathogen, a hypothesis that warrants testing.

Three protein spots whose intensities were reproducibly higher in the mycelial protein extract of the Ontario isolate identified as hypothetical proteins with reasonably good scores contain conserved 20S proteasome component beta 1 domain with expected value 1e-131 (Spot# 16; Figs. 3-3B & 3-4C; Table3-1), conserved proteasome component precursor domain with expected value 1e-99 (Spot# 17; Figs. 3-3B & 3-4C; Table3-1) and conserved malate dehydrogenase, mitochondrial precursor with expected value 3e-168 (Spot# 18; Figs. 3-3B & 3-4C; Table3-1), respectively. The proteasome is a multi-catalytic proteinase complex and has an ATP-dependent proteolytic activity, resulting in the breakdown of a damaged protein or peptide by hydrolysis of its peptide bonds, initiated by the covalent attachment of ubiquitin moieties, to the protein (Djaballah *et al.*, 1993). These two (Spots # 16 & 17) proteins were found to be increased in the more virulent isolate (at least 2-fold)

compared to the less virulent UAMH7476 isolate. The precise meaning of this increase is unknown at this time and warrants further investigation.

As mentioned earlier, one of the hypothetical protein spots (Spot# 18; Figs. 3-3B & 3-4C; Table 3-1) detected as increased in the Ontario isolate contains a highly conserved malate dehydrogenase domain, an important enzyme in the tricarboxylic acid (TCA) cycle. In a study similar to our current one, a comparative proteome-level analysis between two strains of *Botrytis cinerea*, revealed several protein spots identified as MDH in a more virulent strain when compared to a less virulent strain (Fernandez-Acero *et al.,* 2007). Previously, Fernandez-Acero *et al.,* (2006) had suggested a role for MDH as a pathogenicity factor in *Botrytis cinerea*, which may also be the case in the virulent isolate of *A. brassicae*.

In addition to this, another protein observed to be more abundant in the Ontario isolate was a serine protease (Spot# 15; Figs. 3-3B & 3-4C; Table 3-1). Serine proteases belong to a class of enzymes which cleave the peptide bonds in proteins and are characterized by the presence of a serine residue in the active site of the enzyme. Serine protease acts as a virulence factor in the fungal endoparasites *Verticillium suchlasporium* of the cyst nematodes, degrading certain cyst nematode proteins (Lopez-Llorca *et al.,* 1988 and 1990). Serine protease HtrA has also been reported to influence the expression of two virulence factors in the Gram-positive bacterium *Streptococcus pyogenes* (Lyon and Caparon, 2004). Ahman *et al.,* (2002) studied the effects of the deletion of the serine protease *PII* gene from the nematophagous fungus *Arthrobotrys oligospora* which resulted in limited pathogenicity on the nematodes while overexpression of this gene corresponded to a higher capacity to kill nematodes,

suggesting that the *PII* serine protease is a potential pathogenic factor in this fungus. Proteases are also known to contribute to several functions in various other pathogenic bacteria including biogenesis of virulence, cleavage of key host proteins for modulation of host responses and control on protein quality (Collin and Olsen, 2003; Gottesman, 1996; Gottesman *et al.*, 1997). For example, proteins of the HtrA family function as serine protease in Gram-negative bacteria by degrading exported proteins that are misfolded or aggregated and hence play an important role in pathogenesis (Clausen *et al.*, 2002; Cortes *et al.*, 2002). Therefore, the serine protease identified as more abundant in the more virulent isolate of *A. brassicae* may contribute to the biogenesis of virulence factors in this pathogen which may be important for the enhanced virulence.

Another protein spot exhibiting increased abundance in the more virulent Ontario isolate was enolase. Two abundant proteins (Spots# 13 & 14; Figs. 3-3B & 3-4C; Table 3-1) were identified as enolase in this isolate. Enolase (2-phospho-D-glycerate hydrolase) is an ubiquitous enzyme that catalyzes the interconversion of 2-phospho-D-glycerate and phosphoenolpyruvate in the glycolytic pathway. This enzyme was also observed as an extracellular protein secreted by the fungal pathogen *Fusarium graminearum* and was predicted to have a role in pathogenesis due to its role in pathogenicity of some animal pathogenic fungi (Paper *et al.*, 2007). Furthermore, surface enolase is also considered to be a virulence determinant in the Gram-positive bacterium *Streptococcus pneumoniae* (Pancholi and Fischetti, 1998). Moreover, Jong *et al.*, (2003) reported enhanced invasion of human brain endothelial cells as a result of binding of *C. albicans* enolase to plasminogen. Recently,

alternative functions for this enzyme have been identified; for example it has been reported that it functions as a molecular chaperone in tRNA mitochondrial targeting in *Saccharomyces cerevisiae* (Entelis *et al.*, 2006). Because of existing reports in the literature that enolase plays important roles in pathogenesis in the case of several animal pathogens, and because of the possibility of additional roles for this glycolytic enzyme, it is conceivable that the increased enolase levels contributes to the observed increased virulence of the Ontario isolate.

Table 3-1: Details of proteins identified from whole proteome of UAMH 7476 and Ontario isolates of fungal pathogen *Alternaria brassicae*.

Spot #	Protein	MS/MS ESI-Q-ToF			[d]GI#	[e]Status
		[a]PM %	[b]Score	[c]Sequence		
UAMH7476 (less virulent)						
1	Hypothetical protein SNOG_14879 [*Phaeosphaeria nodorum* SN15]	6	19 (>43)	R.HLVENDLVGVEMMSVE HAVR.D	111056611	Unique
2	Hypothetical protein SNOG_09590 [*Phaeosphaeria nodorum* SN15]	7	106 (>40)	R.LVVLLNK.D R.GQLVFGGR.A	111061735	Unique
3	Hypothetical protein GSU2332 [*Geobacter sulfurreducens* PCA]	3	52 (>52)	R.IGALEEYNR.R	39997429	2.9 ± 1.0↑
4	Proteasome component Prs3, putative [*Aspergillus fumigatus* Af293]	5	51 (>43)	K.GALYSYDPVGSYER.E	70991375	13.1 ± 3.7↑
5	BSP2 [*Cryptococcus neoformans* var. grubii]	2	23 (>42)	R.HSRPKLMEAYLDLLGAV AK.G	56566267	8.8 ± 2.4↑
6	Hypothetical protein Kpol_2001p19 [*Vanderwaltozyma polyspora* DSM 70294]	2	22 (>43)	K.LINGVQEVIVNK.I	156846981	34.1 ± 23.0↑
ONTARIO (more virulent)						
7	Hypothetical protein MGG_06837 [*Magnaporthe grisea* 70-15]	11	54 (>43)	K.GEIVDLYVPR.K	39977905	Unique

8	EST1023631 FvM [*Gibberella moniliformis*] cDNA clone FVMA282	7	29 (>52)	R.AFGGDPDKVTIWGESAG AMSVD	70707840	Unique
9	Predicted protein [*Coprinopsis cinerea* okayama7]	4	24 (>43)	K.AIPNPLLNALR.F	116505305	Unique
10	Hypothetical protein [*Aspergillus fumigatus*]	2	24 (>43)	R.ATVDAIEDSAGKTGR.Y	41581211	Unique
11	Hypothetical protein SNOG_03354 [*Phaeosphaeria nodorum* SN15]	10	134 (>41)	R.EESALNMFR.Q K.TVTGVDIAPNSGK.I K.TVHQNTINTIR.S	111067439	Unique
12	Nascent polypeptide-associated complex subunit beta (NAC-beta) (Beta-NAC) [*Phaeosphaeria nodorum*]	13	88 (>41)	K.SDGNVIHFSAPK.V K.LAESYQSMQK.E	121920393	2.2 ± 0.6↑
13	Enolase (2-phosphoglycerate dehydratase) [*Alternaria alternata*]	5	117 (>41)	K.DQSAVDAFLNK.L K.IAMDVASSEFYK.A	14423684	5.5 ± 1.4↑
14	Enolase (2-phosphoglycerate dehydratase) [*Alternaria alternata]*	16	285 (>42)	R.AIVPSGASTGSHEACELR .D K.AVANVNDTIAPALIK.E K. K.IAMDVASSEFYK.A DQSAVDAFLNK.L K.KYDLDFK.N K.SCNALLLK.V	14423684	2.0 ± 0.3↑
15	Serine protease 2 [*Pyrenopeziza brassicae*]	3	75 (>42)	K.GSAANMSLGGGK.S R.AYFSNYGK.C	17385556	3.9 ± 1.2↑
16	Hypothetical protein SPBC4C3.10c [*Schizosaccharomyces pombe* 972h]	4	66 (>41)	K.DGVILAADSR.T	19113087	2.2 ± 0.5↑
17	Hypothetical protein SNOG_03608 [*Phaeosphaeria nodorum* SN15]	22	211 (>41)	K.LTQVHDTIWCCR.S R.HGGQVYSIPLGGSLHK.Q R.HLYLPDQKYDGPGK.Q K.WDGSSGGVIR.M R.TTTGAYIANR.V	111067693	2.6 ± 0.7↑

18	Conserved hypothetical protein [*Botryotinia fuckeliana* B05.10]	13	225 (>41)	R.DDLFNTNASIVR.D R.VQFGGDEVVQAK.D K.DGAGSATLSMAMAGAR .M R.MAESLLK.A	154309029	24.8 ± 16.0↑

[a]Percent sequence coverage.

[b]Mascot score for the most significant hit.

[c]Sequence of the peptide(s) that were matched based on the MS/MS fragmentation patterns.

[d]GI number for the proteins generated by the Mascot search.

[e]Status of a protein spot i.e. unique or abundant in either of the isolate.

3.4. Concluding remarks

Our proteomics-based investigation into the differences at the level of the proteome between two different isolates of *A. brassicae* exhibiting differences in virulence has revealed the identities of several proteins that may potentially be responsible for increased virulence of the Ontario isolate. These proteins include the actin related protein 2/3 complex, proteasome subunit, serine proteases as well as enolase. Many of these proteins were identified as hypothetical proteins from other organisms because of the lack of genome information for *A. brassicae*. However, our study has now established that at least some of these hypothetical proteins could be involved in vital processes including mediating virulence towards the host. Although this study focused on the proteome and it is possible that the differences in virulence may be due to the differences in levels of secondary metabolites (e.g. destruxin B) that are produced by this pathogen. Nevertheless, our study has identified a few proteins that warrant further investigation in this regard. Our current investigation also rationalizes the continued use of proteomics-based techniques to identify novel virulence factors. These could be subsequently targeted, using innovative techniques such as the use of recombinant antibodies, in order to generate resistant varieties.

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4. Towards identifying *Brassica* proteins involved in mediating resistance to *Leptosphaeria maculans*: A proteomics-based approach

4.1. Introduction

Several *Brassica* species such as *Brassica napus*, *B. juncea*, *B. rapa* and *B. oleracea* are important agricultural and horticultural crops (Labana and Gupta, 1993), and are widely cultivated. Phoma stem canker, caused by *Leptosphaeria maculans*, is one of the most devastating and economically important diseases of canola, and losses up to $50 million (Canadian) annually have been attributed to blackleg disease since it was first identified in 1997 (Gugel and Petrie, 1992; Juska *et al.*, 1997). Blackleg infections may occur on cotyledons (Bokor *et al.*, 1975), leaves (Hammond *et al.*, 1985; Barbetti and Khangura, 2000), hypocotyls and stems (Kharbanda, 1993) from the seedling to pod-set stages. Hyphae germinating on cotyledons and leaf surfaces of the plant attack leaf tissues through stomata or existing wounds and initially colonize the mesophyll tissue of leaves and then grow systemically through the vascular tissue to the stem base (Hammond *et al.*, 1985; Hammond and Lewis, 1987). It is also well known that development of severe crown cankers occurs from phoma leaf spots on leaves and therefore, the interaction between the fungus and leaves forms a crucial stage in the disease cycle (Hammond and Lewis, 1986; West *et al.*, 2001).

Several strategies, including chemical treatments are employed for the control of blackleg disease in canola. However, these approaches are not environmentally

sustainable, and are generally not cost-effective (Kharbanda and Tewari, 1996). Fungicides were found to have low eradicant activity and were only effective for a limited period due to their degradation, leaf expansion and formation of new untreated leaves (West *et al.*, 2001). Although many resistant canola varieties have been generated, many new pathotypes of this fungus have recently been identified, which have evolved to overcome the plants' resistance (Sprague *et al.*, 2006). Moreover, breeding for blackleg resistance has become a big challenge due to the limited understanding of the molecular and biochemical mechanisms involved in these host-pathogen interactions. Developing durable resistance to blackleg disease is therefore of the utmost importance. More detailed molecular insights into the interaction of the crop and the pathogen, especially in relation to the differences between compatible and incompatible interactions, might allow the rational development of resistant crops via genetic engineering. Proteome analysis has proven to be a useful tool in characterizing plant-pathogen interactions at the molecular level (Mahmood *et al.*, 2006, Sharma *et al.*, 2007; Kav *et al.*, 2007) such as 2-DE technique which aids the detection of differentially expressed proteins between pathogen-challenged and unchallenged plants. Although transcript profiling has also been used extensively for characterizing global changes in gene expression (Yang *et al.*, 2007), there is sometimes a poor correlation between proteomic and transcriptomic data, due in part to the inability of transcriptomics to detect the influence of post-transcriptional events on gene expression (Gygi *et al.*, 1999).

The relationship between different *Brassica* species was demonstrated by U (1935) suggesting that *B. napus* (AACC) is an amphidiploid with the AA genome

derived from *B. rapa* and the CC genome contributed by *B. oleracea*. *B. carinata* (BBCC), a wild relative of canola, is also an amphidiploid having evolved from *B. nigra* (BB) and *B. olearacea* (CC). B-genome containing Brassicaceae such as *B. carinata* (BBCC), *B. juncea* (AABB) and *B. nigra* (BB) are known to have high levels of resistance to blackleg (Sacristan and Gerdemann, 1986; Sjödin and Glimelius, 1988; Gugel *et al.*, 1990) and therefore have been used as a source of resistance genes by plant breeders to generate blackleg-resistant varieties of canola (Rimmer and van den Berg, 1992; Roy, 1984). It is important to note that while different *B. juncea* lines were observed to be resistant to a virulent *L. maculans* isolate from Canada (Keri *et al.*, 1997), the same lines were found to be susceptible to Australian isolates of *L. maculans* (Kutcher *et al.*, 1993).

Although canola varieties that show resistance to blackleg are currently available, the appearance of newer pathogenic isolates can easily result in a widespread breakdown of resistance that would make most currently available commercial varieties vulnerable. It is therefore critical to identify and exploit alternative sources of resistance to blackleg. Indeed, *B. carinata* has been observed to display a high degree of resistance to both Canadian and Australian isolates of *L. maculans*, both at the seedling and adult stages (Purwantara *et al.*, 1998; Marcroft *et al.*, 1999). However, neither genomic nor proteomic analysis of this important species has yet been performed and molecular details of events that mediate resistance are not known. We therefore decided to investigate the proteome-level changes in the resistant *B. carinata* upon inoculation with *L. maculans* and compared the responses with those of susceptible *B. napus*. Leaf proteome changes were monitored at various

time points following pathogen challenge in both species because of the importance of leaf-fungal interactions for subsequent disease progression culminating in stem lesions. Our investigation led to the identification of a total of 51 proteins (27 in *B. carinata* and 24 in *B. napus*), which provide additional insights into the molecular responses occurring in these two lines during incompatible and compatible interactions.

4.2. Experimental procedures

4.2.1 Plant material and fungal inoculation

In this study, three courses of independent experiments during different time-spans were conducted as follows, each accounting for one biological replicate. Seeds of *B. carinata* and *B. napus* were germinated and grown in plastic trays (7.5 cm x 5 cm; 2 seeds per insert) containing Metro Mix ® 290 (Grace Horticultural Products, Ajax, Ontario, Canada) consisting of vermiculite and peat moss. Plants were grown in the greenhouse (22 °C day/18 °C night; 16 h photoperiod) for 18 days and fertilized with 200 ppm Peters® 20N-20P-20K (Nitrogen-Phosphorus-Potassium) once in 2 weeks.

A highly virulent (PG-2) isolate of *L. maculans* (77-33), which was used as a source of inoculum was kindly provided by Dr. J. P. Tewari, and seeds of both *Brassica* species were obtained from the canola breeding program, Department of Agricultural, Food and Nutritional Science, University of Alberta. A suspension of *L.*

maculans spores in water was prepared by scraping mycelia and spores from plates of actively growing fungal cultures and filtering the suspension through 4-layers of cheesecloth to remove the mycelia. The suspension was centrifuged at 2000 x g for 5 min, the supernatant was discarded and the pelleted spores were washed twice with water to remove remaining metabolites of the media. Washed spores were resuspended in deionized water containing 0.05% Tween-20 (also known as polysorbate 20 used as surfactant), and the spores in this suspension were counted using a haemocytometer and concentration was adjusted to 1 x 10^7 spores/mL. This suspension was used to inoculate 18 day-old seedlings that had been placed in a humidity chamber (100% RH) for 24 h prior to inoculation. The inoculation was performed by gently wounding the true leaves (2 leaves/plant) with a pipette tip and placing 15 µL of spore-suspension at the wounding site. For mock-inoculated controls, 15 µL of sterile deionized water containing 0.05% Tween-20 was placed on the wounding site. The plants were returned to the humidity chamber for another 24 h for the establishment of the fungus, after which they were placed in the greenhouse.

In each biological replicate (64 plants/treatment/species), we collected above ground tissues from 10 randomly selected seedlings from each treatment i.e., pathogen-inoculated or mock-inoculated control groups at each time point (6, 12, 24, 48 and 72 h) post-inoculation. Leaf tissues from these 10 plants for each time point were pooled, flash frozen in liquid nitrogen and stored at -80 °C until protein extraction. The disease symptoms were evaluated 15 days post-inoculation on a total of 14 plants/treatment/species in each independent biological replicate, and the morphological observations were recorded.

4.2.2. Histological studies

Infected leaves and wounded, water-treated controls containing a portion of the inoculated region were cut into small (5 mm x 10 mm) pieces, and fixed in 50 mM phosphate buffer (pH 7.2) containing 4% para-formaldehyde under vacuum at 4 °C overnight. The tissue pieces were subsequently dehydrated in a graded ethanol series, changed to toluene and infiltrated with Paraplast® using a Fisher Histomatic Tissue Processor (Model 166). Paradermal sections (5µm thickness) were cut on an AO Rotary microtome (Spencer 820), affixed to the glass slides, deparaffinated with toluene, rehydrated in 50% ethanol and stained with Aniline blue in lacto-phenol for 10 min (Larone, 1995). Stained sections were rinsed with water (3 x 3 min each), counterstained with acidified Eosin Y (prepared in 80% ethanol) for 1 min (Dougherty and George, 1981), dehydrated using a series of graded ethanol and toluene and mounted with DPX® (Electron Microscopy Sciences, Hatfield, PA, USA) mounting medium. The sections were viewed with a Leica DM *RXA* microscope (Leica Microsystems, Wetzlar, Germany), analyzed using Macrofire™ software (Optronics, Goleta, CA, USA) and photographed with an Optronics digital camera (Optronics). For fluorescence microscopy, the sections were stained with 0.1% berberine (prepared in distilled water) for 1 h, counterstained with 0.5% Aniline blue for 10 min and mounted in 50% glycerol containing 0.1% ferric chloride (Brundrett *et al.,* 1988). The sections were viewed under excitation at 365 nm and emission at 420 nm and photographed as described previously.

4.2.3. Protein extraction

Pooled leaf tissues from 10 plants were ground to a fine powder using liquid nitrogen and the proteins were extracted as previously described (Sharma *et al.*, 2007). Briefly, the tissue powder (350 mg) was homogenized in 1.5 mL of acetone containing 10% (w/v) trichloroacetic acid (TCA) and 0.07% dithiothreitol (DTT). This extract was incubated at -20 °C for 1 h, and centrifuged at 15,000 g for 15 min at 4 °C, and the pellet was washed by resuspension in 1 mL of ice-cold acetone containing 0.07% DTT and centrifuged as described above. The pellet was washed four more times by resuspending in ice-cold acetone containing 0.07% DTT followed by centrifugation as previously described, dried *in vacuo*, resuspended in 450 µL rehydration/sample buffer (Bio-Rad, Canada) containing 0.1% tributylphosphine (TBP) and incubated overnight at 4 °C. After the overnight incubation, the extracts were vortexed vigorously, centrifuged as described earlier and the supernatants were transferred to fresh tubes. The concentration of the protein in the supernatant was determined using a modified Bradford assay (Modified Bio-Rad Protein Assay; Bio-Rad, Canada). Protein-extracts from each of the three independent inoculation experiments were prepared twice from pooled leaf tissue and samples were stored at -20 °C until 2-DE was performed.

4.2.4. Two-dimensional gel electrophoresis (2-DE)

For two-dimensional gel electrophoresis, we accounted for technical variability downstream of protein extraction by extracting protein from each sample twice as described above. Our experimental design for proteomics, consisted of three biological replicates, five different time points (6, 12, 24, 48 and 72 h) per replicate, two gels for each time point (from two independent protein extracts) for both control and pathogen-inoculated plants, and two different species for a total of 120 (2-DE) gels.

Isoelectric focusing (IEF) of protein samples in the first-dimension as well as separation by SDS-PAGE in the second dimension was performed as previously described (Sharma *et al.*, 2007). Briefly, 500 µg of protein in 300 µL of rehydration/sample buffer (Bio-Rad) was used to passively hydrate 17 cm IPG strips (pH 4-7, linear; Bio-Rad) and isoelectric focusing was performed using a Bio-Rad PROTEAN IEF unit. The focused IPG strips were equilibrated in buffers containing 6 M urea, 2% SDS, 20% glycerol, 0.37 M Tris-HCL (pH 8.8) and 130 mM DTT for the reduction of sulfhydryl groups followed by a second equilibration in the same buffer (no DTT) containing 135 mM iodoacetamide, for the alkylation of the reduced sulfhydryl groups prior to second dimension SDS-PAGE. Second dimension electrophoresis was carried out on 13% polyacrylamide gels (20 cm × 20 cm, 1 mm thickness; containing 0.1% SDS, 0.1% ammonium persulfate, 0.375 M Tris-HCL pH 8.8, 13.3% acrylamide/bis and 0.04% TEMED) using a PROTEAN II xi system (Bio-Rad) at 45 V/gel until 2 h after the dye front reached the bottom of gel. Gels were

stained overnight with 200 mL of colloidal Coomassie stain (CBB; Invitrogen) as per manufacturer's instructions and destained in water till the protein spots were clearly visible. For each time-point, six gels obtained from the two technical repetitions of protein extractions from the three independent biological replicates were scanned using a GS-800 calibrated densitometer (Bio-Rad).

4.2.5. Image analysis and statistical treatments

Six gels (two technical x three biological replicates for each time point) constituted an independent match-set in PDQuest software (Bio-Rad). We then used the automated spot detection feature of the PDQuest software to detect and match spots between gels of mock-inoculated control and pathogen-challenged samples. Addition of spots missed by this process and deletion of artifacts identified as spots was performed manually. In order to identify those protein spots exhibiting statistically significant changes in abundance as a result of pathogen challenge we first used the built-in Student's *t*-test module in PDQuest at $p<0.05$ on each match-set, which aimed to test the significance of changes in abundance of a given spot at a given time-point. These PDQuest results were further verified by extracting the intensities of every spot exhibiting statistically significant modulation of intensity and performing additional statistical analysis as described below.

Fold changes in intensities for each spot at every time point were calculated across biological and technical replicates. These fold changes were then used to calculate the expression ratio (ER) between mock-treated control and pathogen-

challenged samples. These ER values were then transformed to the log_2scale to generate log_2 (ER) for every spot across time points. This was performed in order to permit cross-time point comparisons and to make the data more suited to parametric statistical tests. We then performed a one-sample Student's *t*-test on the ER using SAS (Statistical Analysis System; version 9.1; SAS Institute Inc., Cary, NC) with a null hypothesis of $\{H_0: Log_2(ER)=0\}$. We also tested this hypothesis against the alternate hypotheses $\{H_1: Log_2(ER)<0\}$ or $\{H_1: Log_2(ER)>0\}$ depending on the direction of the changes observed for each spot. Additionally, in order to compare changes in ER across different time points and to investigate significance of the trend, we used generalized linear model (GLM) procedure for analysis of variance (ANOVA) across the time points for a given spot. In order to achieve this, we introduced time-point as a fixed factor, biological replicates and technical replicates as random factors. ANOVA was then followed by Duncan's multiple range test ($p<0.05$) in order to identify time-points differing from one another with respect to ER.

4.2.6. Mass Spectrometry

Spots exhibiting reproducible intensity changes following pathogen challenge were excised from the gels using a sterile scalpel to avoid any cross contamination with other spots and the proteins were identified using ESI-ion trap MS/MS. Processing of the gel pieces was performed in a fully automated fashion on a Mass Prep Station (Micromass, UK). We performed peptide identification using an Agilent 1100 series HPLC online with an XCT Ion Trap (Agilent Technologies) by using an

autosampler to inject 18 uL of each sample onto an enrichment column (Zorbax 300SB-C18 5 µm 5 x 0.3 mm) that connected to a second column (Zorbax 300SB-C18 5 µm 150 x 0.3 mm) in a peptide-separation gradient that started at 85% solvent A (0.1% formic acid in H_2O) and ended at 55% solvent B (0.1% formic acid, 5% H_2O in ACN) over a span of 42 min. This was followed by 10 min of 90% solvent B to cleanse the columns before returning to 97% solvent A for the next sample. The MS ran a 300-2200 m/z scan followed by MS/MS analysis of the most intense ions. Raw spectral data was processed into Mascot Generic File (mgf) format using the default method in the ChemStation Data Analysis module and ion searches were completed in MASCOT (Matrix Science, UK; 1999) with the search parameters of: NCBI non-redundant (nr) database, 2 missed cleavages, Carbamidomethyl fixed modification, 2 Da peptide tolerance, 0.8 m/z MS/MS tolerance, peptide charge +1, 2+ and 3+, with no precursor m/z. MS/MS data was used to identify proteins through a search of the NCBI non-redundant database using Mascot Daemon (Matrix Science, UK).

4.2.7. Quantitative Real Time PCR (q-RT-PCR)

q-RT-PCR using the SYBR green method was performed for selected genes in order to investigate changes in mRNA levels. *B. napus* gene sequences were used to design primers using Primer Premier 3 software (Applied Biosystems Inc., CA, USA) to generate amplicons of 80 bp size (genes and primer sequences are shown in Table 4-1). These primers were checked using nucleotide BLAST for their specificity to the genes whose accession numbers are provided in Table 4-1. Total RNA was extracted

using a QIAGEN RNeasy Plant Mini Kit (Qiagen, ON, Canada) from 100 mg of leaf tissue obtained from water and pathogen-inoculated plants of both species at 6, 12, 24, 48 and 72 h following infection. Reverse transcription of total RNA (50 ng) was carried out using iScript cDNA synthesis kit (Bio-Rad, CA, USA). The q-RT-PCR was performed with 2.5 µL of 10x diluted cDNA as template, 2.5 µL of each primer and 5.0 µL SYBR green Master Mix (containing Tris (pH 8.3), 3 mM $MgCl_2$, 50 mM KCl, 0.01% Tween 20, 0.8% glycerol, 2% dimethyl sulfoxide (DMSO), 0.2 mM dNTPs, 1x ROX, 0.25x SYBR, 0.03 units/µL Platinum Taq) in a total volume of 10 µL. PCR analysis was performed using an ABI 7500 Sequence detector (Applied Biosystems Inc., CA, USA) as per the manufacturer's instructions. Relative expression of all genes was determined using the delta-delta method utilizing the formula: relative expression = $2^{-[^{\Delta Ct\ sample} - ^{\Delta Ct\ control}]}$ (Livak and Schmittgen, 2001) where Ct refers to the threshold cycle, sample stands for the gene of interest and control indicates the endogenous house-keeping gene (actin). The expression level of control (un-inoculated) plants was equated to 1 for each time-point and the relative expression in the inoculated plants was normalized against these levels. The reactions were performed in triplicates.

Table 4-1 Sequences of the primers used for q-RT-PCR analysis

Gene	Accession #	Primers
Type-II-peroxiredoxin (*Brassica rapa*)	AF133302	F-5'- GGAGCTGAAGTCAAAGGGTGTT-3' R-5'-TCTTCCCCCATGCCTTCAT-3'
Germin-like protein (*Brassica napus*)	U21743	F-5'-TTCTTGACTTTGCGCTATTTGC-3' R-5'-GACTGTAGCAGGCGGTAGGAA-3'
Ascorbate peroxidase (*Brassica napus*)	Y11461	F-5'-CTTCTTCAGCTCCCCTCTGACA-3' R-5'-TCGTCAGCAGCGTATTTCTCA-3'
2-cys-peroxiredoxin (*Brassica napus*)	CD814109	F-5'-GAGGGCTCGGTGATCTGAAC-3' R-5'-AGGGATGAGCACACCAAAAGA-3'
Actin (*Brassica napus*)	AF111812	F-5'-ACGAGCTACCTGACGGACAAG-3' R-5'-GAGCGACGGCTGGAAGAGTA-3'

4.2.8. Western Blot Analysis

We verified the identification of germin-like protein using Western blot analysis with the same protein extract that was used for 2-dimensional gel electrophoresis. Following two- dimensional gel electrophoresis, proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad) using the Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad) according to the manufacturer's recommended procedures. Briefly, gels were equilibrated for 25 min in Bjerrum and

Schafer-Nielsen transfer buffer containing 48 mM Tris-base, 39 mM glycine, 20% methanol, 1.3 mM SDS, pH 9.2. The PVDF membrane was moistened in methanol for 1 min and equilibrated in transfer buffer along with thick filter paper for 10 min. The transfer was performed at 15 V for 30 min and the membrane was incubated overnight in blocking buffer containing 10% (w/v) Bio-Rad blotting grade non-fat dry milk in 1x TBS buffer (154 mM Nacl and 50 mM Tris-base, pH 7.5). The membrane was washed with 1x TTBS buffer (TBS with 0.05% Tween-20) for 10 min followed by incubation in primary antibody (AtGLP; 1:20,000) solution for 1h. After washing the membrane three times with 1x TTBS buffer for 5 min each to remove unbound primary antibody, the membrane was incubated in a solution containing goat anti-rabbit IgG (H&L) Horseradish Peroxidase secondary antibody for 1h. After washing the membrane as before, the Western blot was developed using TMB (3, 3', 5, 5'-tetramethylbenzidine) membrane staining substrate solution as per the manufacturer's instructions (Vector Laboratories, Burlingame, CA, USA). The image was scanned using the Alpha Innotech Fluorchem-SP machine (Alpha Innotech Corporation, San Leandro, CA, USA) and the spot density of germin-like protein was calculated with the spot density tools of the Alpha Ease FC machine (Alpha Innotech Corporation, San Leandro, CA, USA). This experiment was repeated three times with 3 independent biological replicates of un-inoculated and *L. maculans*-inoculated *B. napus* and *B. carinata*. The antibody used in these experiments was generously provided by Dr. François Bernier, Institut de Biologie Moléculaire des Plantes, Strasbourg cedex, France.

4.3. Results and Discussion

4.3.1. Responses of *B. carinata* and *B. napus* to *L. maculans*: morphological and histological characterization

The morphological appearance of *B. napus* (cv. Westar) and *B. carinata* used in the present study 15 days after inoculation with *L. maculans* is shown in Fig. 4-1. As expected, the blackleg-susceptible *B. napus* displayed severe disease symptoms such as chlorosis and necrosis around the inoculated leaf area with irregular margins and pycnidial growth over collapsed tissue (Fig. 4-1A & B) whereas the leaves of the blackleg-resistant *B. carinata* appeared devoid of any chlorotic/necrotic tissue and appeared to be totally resistant to the invading pathogen (Fig. 4-1C & D). There were no visible symptoms in the susceptible species (*B. napus* cv. Westar) up to 7 days post-inoculation, after which the symptoms appeared and spread rapidly until the plants were photographed on day 15.

The interaction of *L. maculans* with both the *Brassica* species was also examined using light microscopy at 8 days post-inoculation. This day was chosen for histological examination since no visible symptoms were observed until this time point. Histological observation of *B. carinata* and *B. napus* mock-inoculated controls, as well as the inoculated tissues of *B. carinata* revealed the presence of dead and injured cells at the site of wounding (Figs. 4-2A, B & C). The absence of any visible fungal material in the samples obtained from *B. carinata* infected with *L. maculans* was consistent with its resistant phenotype. Tissue from *L. maculans*-challenged *B.*

napus plants, on the other hand, revealed the presence of dead and lysed cells, extensive fungal mycelial growth and the presence of pycnidia (Fig. 4-2D). Once again, our histological observations with the *L. maculans*-challenged *B. napus* tissue were consistent with its susceptible phenotype and provide microscopic evidence for the successful colonization of the susceptible host, and the formation of pycnidia to serve as an additional inoculum source for the subsequent spread of the disease. Similar extensive colonization of *B. napus* cv. Westar by invading fungal hyphae and pycnidia formation by *L. maculans* has previously been reported (Li *et al.*, 2004).

The hypersensitive response (HR) is characterized by an oxidative burst, localized cell death, accumulation of cell wall bound phenolics and synthesis of hydrolytic enzymes. The HR is a common strategy employed by plants to resist invading fungal and bacterial pathogens (Dixon *et al.*, 1994). Lignin deposition at the site of contact with the pathogen forms a barrier to further infection. There exists a close relationship between lignin deposition and disease resistance; resistant plants have rapid/enhanced lignin deposition compared to susceptible plants (Nicholson and Hammerschmidt, 1992) and the inhibition of lignification. The inhibition of HR has also been shown to render normally resistant wheat plants susceptible to fungal infection (Moerschbacher *et al.*, 1990).

In order to examine the role of lignification in the resistance of *B. carinata* to *L. maculans*, we stained pathogen-challenged tissue from both *B. napus* as well as *B. carinata* tissue for lignin using Berberine-Aniline blue (Figs. 4-2E, F). In the case of *B. carinata*, our results clearly indicate the presence of a well-defined layer of lignified cells surrounding the site of inoculation with *L. maculans* (Fig. 4-2F) and in

the case of the susceptible *B. napus*, no such lignification around the site of inoculation was observed (Fig. 4-2E). In both cases, tracheids, which are also known to contain abundant lignin, were also intensely stained. The formation of a well defined layer of lignified cells potentially plays an important role in restricting the advancement of the invading fungus thereby limiting its spread through the plant which could be significant for the observed resistance of *B. carinata* to this pathogen.

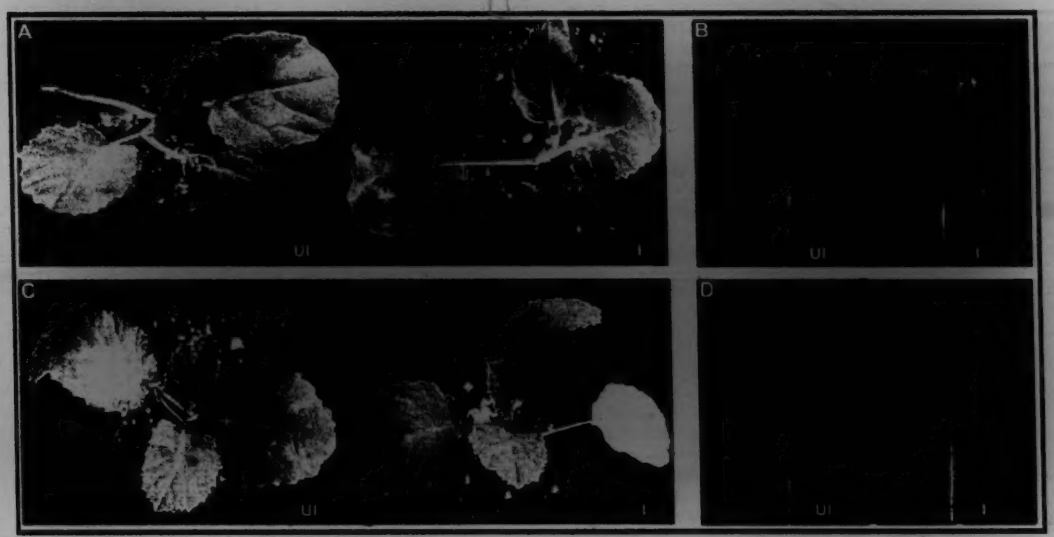


Figure 4-1. The morphological appearance of plants and leaves of *B. napus* and *B. carinata* 15 days following infection. Panels (A) and (B) display chlorosis and necrosis with pycnidia formation around the inoculated area in susceptible *B. napus*. Panels (C) and (D) display total resistance to the disease in resistant *B. carinata*. The labels UI and I refer to un-inoculated (mock-inoculated) and inoculated (pathogen-inoculated) respectively in the figure.

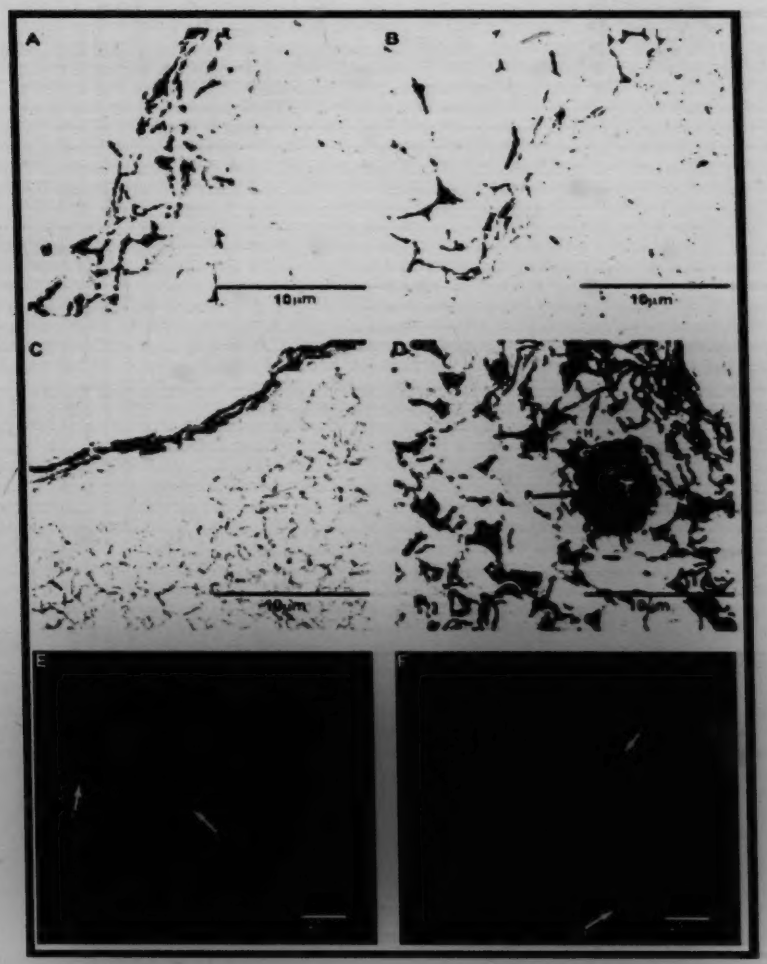


Figure 4-2. Light microscopic images of *B. napus* and *B. carinata*. Panels (A) and (C) show dead and injured cells at the wounding site in mock-inoculated leaves of *B. carinata* and *B. napus* respectively. Panel (B) shows the absence of fungal growth in *B. carinata* while panel (D) displays profuse fungal hyphal growth (H) with the presence of a pycnidium (P) in *B. napus* respectively. For fluorescence microscopy, panel (E) shows no lignification at the site of pathogen inoculation in susceptible *B. napus* whereas panel (F) shows the presence of a well-defined layer of lignified cells around the pathogen-inoculated area in resistant *B. carinata*.

4.3.2. Effect of the pathogen on leaf proteomes

To evaluate the effects of pathogen infection on the leaf proteome of *B. napus* and *B. carinata*, we performed two-dimensional gel electrophoresis (2-DE) at 6, 12, 24, 48 and 72 h after inoculation and representative images are shown in Figs. 4-3A-B. Six independent gels (two from each of the three biological replicates) were analyzed for each treatment (control plants and pathogen challenged) and for each time point separately using the Student's *t*-test feature of the PDQuest software (Bio-Rad). Approximately 300 (274-294) spots were observed to be resolved in the pH range of 4-7 in all the gels for either *Brassica* species. Figs. 4-4 and 4-5 show enlarged images of spots whose levels were significantly altered due to *L. maculans* infection in *B. carinata* and *B. napus*, respectively.

Based on PDQuest analysis, a total of 37 spots were observed to be reproducibly and significantly ($p<0.05$) altered in intensities in *B. carinata* following pathogen-challenge. Among them, 11 spots were observed to be affected at 6 h; 4 at 12 h; 10 at 24 h; 5 at 48 h and 7 at 72 h post-inoculation (Figs. 4-3A & 4-4). All spots were observed to increase in intensity at 6 and 24 h, decreased at 12 and 48 h, whereas 3 increased and 4 decreased at 72 h. In *B. napus*, a total of 27 spots were observed to be altered in intensity in response to the pathogen with 2 at 12 h; 21 at 24 h; 3 at 48 h and 1 at 72 h post-inoculation with the pathogen. Interestingly, in this case, the intensity of only 1 spot was observed to be increased (48 h) whereas the remaining 26 protein spots decreased in intensity in response to the pathogen (Figs. 4-3B & 4-5). It must be noted that with a confidence interval of 95% ($p<0.05$), at the most 15 spots

out of the approximately 300 spots observed on our 2-DE gels might be expected to be false positives. However, in the case of both *B. napus* and *B. carinata* we have observed 27 and 37 spots, respectively whose intensities were altered significantly upon pathogen challenge. Nevertheless, it is reasonable to expect a few false positives among the proteins that we have identified in this study. All these proteins spots (from both *B. carinata* as well as *B. napus* gels) exhibiting statistically significant altered expression ($p<0.05$) were excised from the gels and subjected to mass spectrometry.

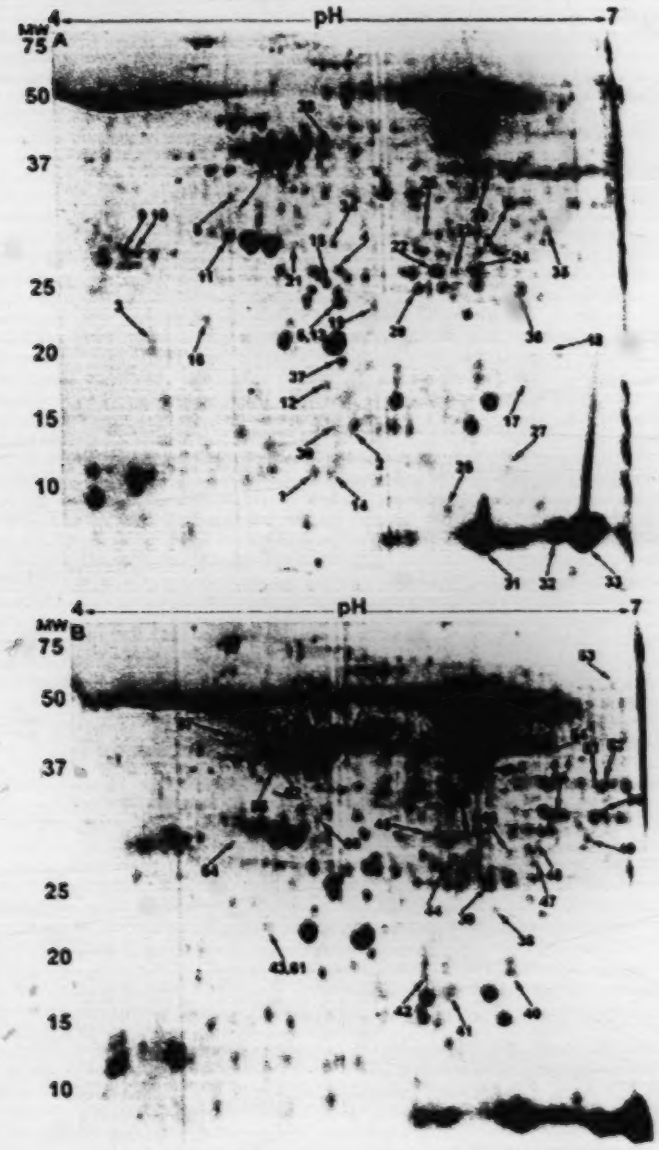


Figure 4-3. CBB- stained images of two-dimensional gels of leaf proteomes of a blackleg-resistant *B. carinata* (A) and blackleg-susceptible *B. napus* (B) at 6 h, 12 h, 24 h, 48 h and 72 h respectively.

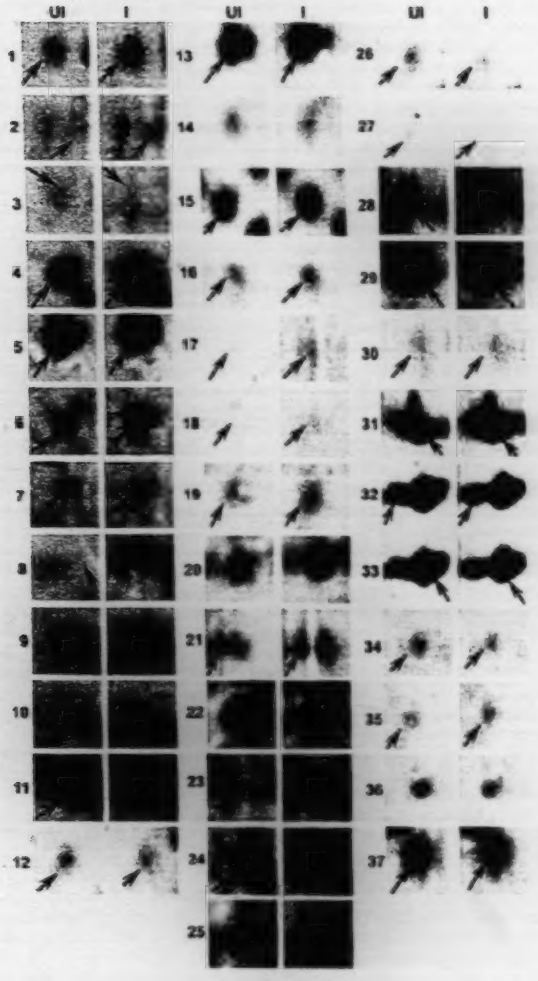


Figure 4-4. Panels show enlarged images of the protein spots affected by pathogen infection in *B. carinata.* The spots from un-inoculated (UI) and pathogen-inoculated (I) leaves are shown in this panel.

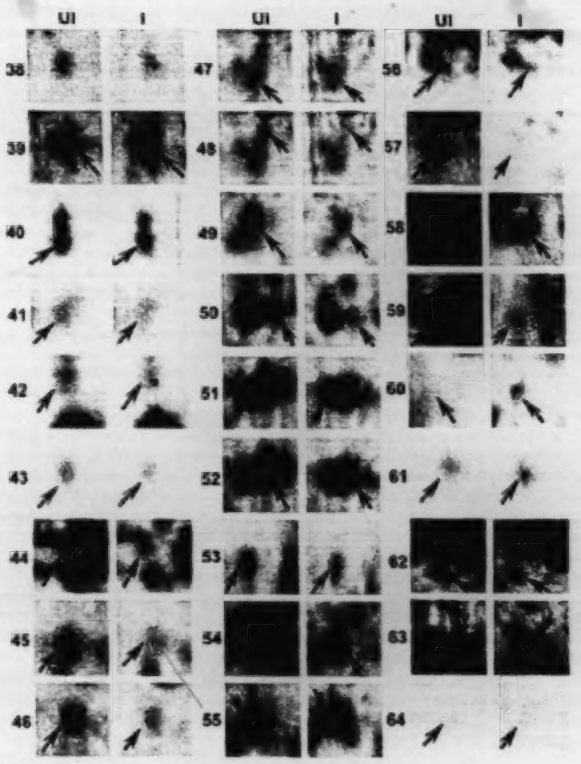


Figure 4-5. Panels show enlarged images of the protein spots affected by pathogen infection in *B. napus*. The spots from un-inoculated (UI) and pathogen-inoculated (I) leaves are shown in this panel.

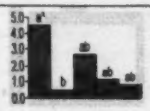
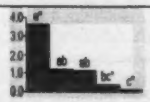
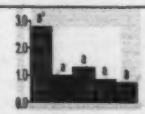
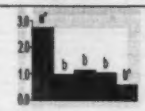
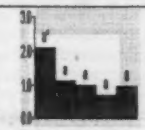
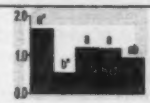
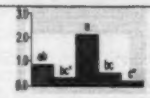
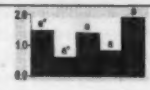
4.3.3. Identities of proteins and analysis of changes in abundance

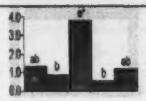
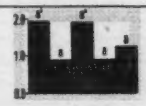
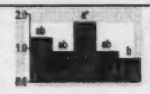
As mentioned previously, upon PDQuest analysis, a total of 37 and 27 protein spots (total 64) were found to be differentially expressed in *B. carinata* and *B. napus,* respectively, at the various time-points that were investigated following pathogen-challenge and were subjected to MS/MS analysis. We were able to identify a total of 62 proteins however, only 51 among those generated "hits" with two or more unique peptides and only their identities are presented in Table 4-2. Among these, 7 proteins were identified at 6; 2 at 12; 8 at 24, 4 at 48 and 6 at 72 h in *B. carinata* after challenge with *L. maculans.* As indicated earlier, no proteins were observed to be significantly affected in intensity at 6 h following pathogen-challenge in the case of *B. napus*. In this species, the identities of two proteins at 12; 19 at 24; 3 at 48 and 0 at 72 h post pathogen-challenge are listed in Table 4-2.

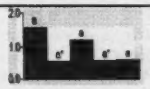
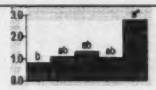
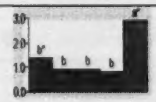
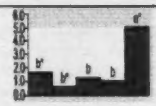
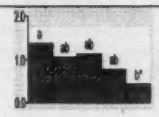
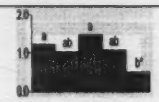
As described earlier, we also performed statistical analysis to verify the PDQuest results and also to compare the modulation of spot intensities across different time points using $\log_2$ [expression ratio (ER)]. This analysis revealed that there were no significant differences for the most part between the trends observed employing either method of analysis i.e. PDQuest vs. one-sample Student's *t*-test. The only exceptions to this were in the case of spots 23, 28 and 48 where our extended statistical analysis failed to confirm PDQuest results. Additionally, we used the $\log_2$ (ER) to compare changes in protein spot intensities across different time points using ANOVA and Duncan's multiple-range test. Our results indicated that several spots exhibited statistically significant changes in abundance at more than one time point

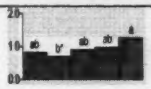
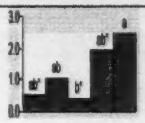
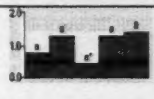
and these results are presented in Table 4-2. For example, the intensities of dehydroascorbate reductase (Spot# 20) and ascorbate peroxidase (Spot# 22) were observed to be significantly increased at the 6 h time point as well in *B. carinata* following pathogen challenge (Table 4-2). In addition, spots 2, 14, 26, 28, 29 and 30 were observed to be significantly down-regulated at more than one time-point in blackleg-resistant *B. carinata* (Table 4-2). In case of the *B. napus* leaf proteome, additional analysis revealed spots 38, 41, 43, 44, 46, 47, 50, 51, 52 and 61 were decreased in their intensities at more than one time point, whereas no protein spot was observed to be up-regulated at more than one time point in this species. Additionally, spots 38, 42, 43 and 60 were identified as increasing in intensity at 48 h following pathogen challenge as a result of this extended analysis (Table 4-2). Interestingly, these spots were identified by the PDQuest analysis as being decreased at earlier time points which was confirmed by our extended statistical analysis (Table 4-2). The proteins identified in this study were classified according to their functions based on information available in the literature and verified using gene ontology in Pfam server (http://pfam.sanger.ac.uk/). The majority of proteins that were identified from both the tolerant and susceptible species are those that are involved in the production and detoxification of reactive oxygen species (ROS), energy or metabolic pathways, protein-folding and signal transduction and are discussed below (Table 4-2).

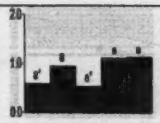
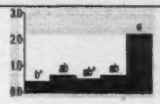
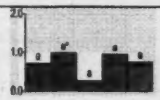
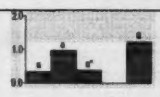
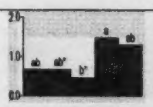
Table 4-2 Details of proteins identified from *L. maculans*-resistant *Brassica carinata* and *B. napus* at various times after challenge with the pathogen

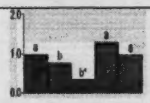
Spot #	[α]Expression ratios	Protein	MS/MS ESI-Q-ToF			[ε]GI #
B. carinata			[β]PM %	[γ]Score	[δ]Sequence	
1		Glycine rich protein [*Arabidopsis thaliana*]	10%	74 (>)50	R.GFGFVTFK.D R.SITVNEAQSR.G	16301
2		Type 2 peroxiredoxin [*Brassica rapa* subsp. pekinensis]	17%	150 (>50)	K.HVPGFIEK.A K.FVADGSGEYTK.L K.LLGLELDLK.D	4928472
7		Putative glyoxalase [*Oryza sativa* (japonica cultivar-group)]	6%	47 (>46)	K.FELIER.G R.EPGPLPGINTK.I	46485858
8		Malate dehydrogenase NAD-dependent [*Brassica napus*]	11%	132 (>50)	K.ALEGADLVIIPAG VPR.K R.DDLFNINAGIVK.N K.LFGVTTLDVVR.V	899226
9		RNA binding / nucleic acid binding [*Arabidopsis thaliana*]	6%	61 (>50)	R.VNAGPPPPK.R R.VSEAEARPPR.R	15228102
10		RNA binding / nucleic acid binding [*Arabidopsis thaliana*]	6%	71 (>49)	R.VNAGPPPPK.R R.VSEAEARPPR.R	15228102
11		33 kDa oxygen evolving protein of photosystem II [*Brassica napus*]	15%	129 (>50)	R.LTYDEIQSK.T K.GTGTANQCPTIDG GSETFSFKPGK.Y K.NAPPDFQNTK.L	5052366
14		Glycine-rich RNA-binding protein 10 [*Brassica napus*]	17%	150 (>46)	R.GFGFVTFK.D R.TITVNEAQSR.G R.TFSQFGEVIDSK.I	544416
15		Triosephosphate isomerase, cytosolic (TIM) [*Secale cereale*]	11%	75 (>46)	K.FFVGGNWK.C K.VAYALAQGLK.V K.VIACVGETLEQR.E	1174749

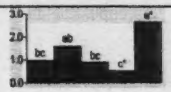
16		2-cys peroxiredoxin-like protein [*Hyacinthus orientalis*]	20%	95 (>47)	R.GLFIIDK.E K.EGVIQHSTINNLGI GR.S K.SGGLGDLNYPLVS DVTK.S	47027073
19		Multicatalytic endopeptidase complex, proteasome precursor, beta subunit [*Arabidopsis thaliana*]	8%	51 (>46)	K.EEAEQLVVK.A R.TVIINSEGVTR.N	21592365
20		Dehydroascorbate reductase [*Lycopersicon esculentum*]	11%	117 (>47)	K.AAVGAPDVLGDC PFSQR.V R.VLLTLEEK.K	66475036
21		PSBO-1 (Oxygen-evolving enhancer 33) [*Arabidopsis thaliana*]	27%	310 (>46)	R.LTYDEIQSK.T K.GTGTANQCPTIDG GSETFSFKPGK.Y K.FCFEPTSFTVK.A R.VPFLFTVK.Q R.GGSTGYDNAVALP AGGR.G K.NTAASVGEITLK.V K.IQGVWYGQLE.	15240013
22		L-ascorbate peroxidase [*Brassica napus*]	15%	160 (>46)	R.LLEPIR.E K.LSELGFADA K.EGLLQLPSDK.A R.LAWHSAGTFDCAS R.T	1890354
23		Ascorbate peroxidase [*Brassica juncea*]	14%	153 (>48)	K.SYPTVSEDYQK.A R.LLDPIR.E K.EGLLQLVSDK.A K.LSELGFADA.	24421231
24		Ascorbate peroxidase [*Brassica juncea*]	12%	70 (>46)	K.LSELGFADA. K.EGLLQLVSDK.A K.SYPTVSEDYQK.A	24421231
25		Ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit [*Leucocroton acunae*]	11%	106 (>47)	K.DTDILAAFR.V K.LTYYTPEYETK.D R.AVYECLR.G	78522553
26		Nucleoside diphosphate kinase 1 [*Brassica rapa*]	17%	114 (>46)	R.GLVGEIICR.F R.KIIGATNPAASEPG TIR.G K.IIGATNPAASEPGT IR.G	19570344
28		NADP-dependant malate dehydrogenase [*Paspalum paniculatum*]	5%	82 (>47)	K.ALNAVASPNVK.V K.SEAELLAEK.K	24370966

29		Malate dehydrogenase/ oxidoreductase NAD-dependent [*Arabidopsis thaliana*]	10%	91 (>46)	K.EFAPSIPEK.N K.VLVVANPANTNALILK.E R.LSVPVSDVK.N	15219721
30		Type 2 peroxiredoxin [*Brassica rapa* subsp. Pekinensis]	12%	73 (>46)	R.FALLIDNLK.V K.FVADGSGEYTK.L	4928472
31		Ribulose bisphosphate carboxylase, small subunit [*Brassica napus*]	48%	335 (>50)	K.EVDYLLR.N RIIGFDNNR.Q R.EHGSTPGYYDGR.Y K.TEYPNAFIR.I K.WIPCVEFELEHGFVYR.E R.YWTMWK.L R.IIGFDNNR.Q R.QVQCISFIAYKPPSFTGA	17850
32		Ribulose bisphosphate carboxylase, small subunit [*Brassica napus*]	32%	255 (>50)	K.EVDYLLR.N RIIGFDNNR.Q R.EHGSTPGYYDGR.Y K.TEYPNAFIR.I K.WIPCVEFELEHGFVYR.E R.YWTMWK.L R.IIGFDNNR.Q	17850
33		Ribulose bisphosphate carboxylase, small subunit [*Brassica napus*]	38%	309 (>50)	K.EVDYLLR.N RIIGFDNNR.Q R.EHGSTPGYYDGR.Y K.TEYPNAFIR.I	17850
34		Hypothetical protein At2g37660 [imported] – [*Arabidopsis thaliana*]	7%	62 (>46)	K.ALFTQVTTK.F K.ALDLASKPEGTGTPTK.D	7485430
35		Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [*Cremastosperma yamayakatense*]	11%	174 (>47)	R.AVYECLR.G K.DTDILAAFR.V K.LTYYTPEYETK.D R.LSGGDHVHAGTVVGK.L R.DLAVEGNEIVR.E	30959094
36		Carbonic anhydrase [*Arabidopsis thaliana*]	11%	109 (>46)	K.YETNPALYGELAK.G K.VENIVVIGHSACGGIK.G	438449

Spot #	[α]Expression ratios	Protein	MS/MS ESI-Q-ToF			[ε]GI #
			[β]PM %	[γ]Score	[δ]Sequence	
B. napus						
38		3-phosphoglycerate kinase [*Hordeum vulgare* subsp. vulgare]	8%	93 (>47)	R.LSELLGIEVK.K K.GVSLLLPSDVVIADK.F	21396683
39		Carbonic anhydrase [*Arabidopsis thaliana*]	20%	191 (>47)	K.EKYETNPALYGELAK.G K.YETNPALYGELAK.G K.YMVFACSDSR.V R.NIANMVPPFDK.V K.VISELGDSAFEDQCGR.C	438449
40		Germin-like protein [*Arabidopsis thaliana*]	8%	102 (>46)	K.NPDQVTENDFAFTGLGK.A K.LKGVLGGTN.	1755154
41		ROC4; peptidyl-prolyl cis-trans isomerase [*Arabidopsis thaliana*]	7%	90 (>46)	K.FEDENFTLK.H R.IYACGELPLDA.-	15228674
42		ROC4; peptidyl-prolyl cis-trans isomerase [*Arabidopsis thaliana*]	7%	57 (>47)	K.FEDENFTLK.H R.IYACGELPLDA.-	21555831
43		Oxygen-evolving enhancer protein 2, chloroplast precursor (OEE2) (23 kDa subunit of oxygen evolving system of photosystem II)] [*Sinapis alba*]	11%	69 (>47)	R.EVEYPGQVLR.Y R.TADGDEGGKHQLITATVNGGK.L	131391
44		Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [*Forchhammeria watsonii*]	7%	99 (>47)	R.ESTLGFVDLLR.D R.DLAVEGNEIIR.E	46325929
45		Ribulose bisphosphate carboxylase large chain precursor (RuBisCO large subunit) [*Brassica oleracea*]	9%	144 (>46)	R.LSGGDHVHAGTVVGK.L R.ESTLGFVDLLR.D R.VALEACVQAR.N R.DLAVEGNEIIR.E	1346967

46		Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [*Pentadiplandra brazzeana*]	6%	142 (>47)	R.VALEACVQAR.N R.DLAVEGNEIIR.E R.ESTLGFVDLLR.D	1054917
47		Ribulose bisphosphate carboxylase large chain precursor (RuBisCO large subunit) [*Brassica oleracea*]	16%	159 (>47)	K.LNYYTPEYETK.D K.DTDILAAFR.V K.ALAALRLEDLRIPPAYTK.T R.FLFCAEAIYK.S R.ESTLGFVDLLR.D R.VALEACVQAR.N R.DLAVEGNEIIR.E	1346967
48		Ribulose bisphosphate carboxylase large chain precursor (RuBisCO large subunit) [*Brassica oleracea*]	16%	136 (>47)	K.LNYYTPEYETK.D K.DTDILAAFR.V K.ALAALRLEDLRIPPAYTK.T R.FLFCAEAIYK.S R.ESTLGFVDLLR.D R.VALEACVQAR.N R.DLAVEGNEIIR.E	1346967
49		Ribulose bisphosphate carboxylase large chain precursor (RuBisCO large subunit) [*Brassica oleracea*]	12%	255 (>47)	K.LNYYTPEYETK.D K.DTDILAAFR.V K.YGRPLLGCTIKPK.L R.AVYECLR.G R.FLFCAEAIYK.S R.VALEACVQAR.N	1346967
50		Ribulose bisphosphate carboxylase large chain precursor (RuBisCO large subunit) [*Polanisia dodecandra*]	15%	160 (>47)	K.DTDILAAFR.V K.ALAALRLEDLRIPPAYTK.T R.FLFCAEAIYK.S R.ESTLGFVDLLR.D R.VALEACVQAR.N R.DLAVEGNEIIR.E	30959098
51		Malate dehydrogenase/ oxidoreductase NAD-dependent [*Arabidopsis thaliana*]	10%	107 (>47)	K.VLVVANPANTNALILK.E K.EFAPSIPEK.N R.LSVPVSDVK.N	15219721
52		Fructose-bisphosphate aldolase [*Arabidopsis thaliana*]	22%	244 (>47)	K.GILAADESTGTIGK.R R.LASINVENVETNR.R K.LFVDILK.E K.EGGVLPGIK.V K.AAQEALYVR.C K.ANSEATLGTYK	15231715

					GDAK.L K.LGDGAAESLHV K.D K.LGDGAAESLHV K.D	
53		Dihydrolipoyl dehydrogenase [*Arabidopsis thaliana*]	13%	239 (>46)	R.GIEGLFK.K K.AIDNAEGLVK.I K.SLPGITIDEK.K R.TPFTSGLDLEK.I K.IVSSTGALSLSE VPK.K R.GALGGTCLNVG CIPSK.A K.IVSSTGALSLSE VPKK.L	15221044
54		Glyceraldehyde-3-phosphate dehydrogenase (NADP) B precursor, chloroplast [*Arabidopsis thaliana*]	9%	70 (>47)	K.IVDNETISVDGK. L K.VLDEEFGIVK.G R.AAALNIVPTSTG AAK.A	81621
57		Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [*Neoregelia schubertii*]	3%	59 (>46)	R.AVYECLR.G R.DLAREGGEIIR.E R.DLAREGCEIIR.E	66802797
58		Glutamine synthetase [*Brassica napus*]	5%	66 (>47)	K.AILNLSLR.H R.TLEKPVEDPSEL PK.W	6966930
59		Ribulose bisphosphate carboxylase large chain precursor (RuBisCO large subunit) [*Salvadora persica*]	8%	197 (>47)	R.AVYECLR.G K.DTDILAAFR.V R.VALEACVQAR.N R.DLAVEGNEIIR.E	1352808
60		VHA-E3; hydrogen-transporting ATP synthase, rotational mechanism / hydrogen-transporting ATPase, rotational mechanism [*Arabidopsis thaliana*]	8%	89 (>46)	K.DLIVQCLLR.L K.IVCENTLDAR.L	15222641
61		Oxygen-evolving enhancer protein 2, chloroplast precursor (OEE2) (23 kDa subunit of oxygen evolving system of photosystem II)] [*Sinapis alba*]	11%	69 (>46)	R.EVEYPGQVLR.Y R.TADGDEGGKHQ LITATVNGGK.L	131391

62		Magnesium-chelatase subunit chlI, chloroplast precursor (Mg-protoporphyrin IX chelatase) [*Glycine max*]	14%	195 (>47)	K.LCLLLNVIDPK.I R.VCGTIDIEK.A K.AFEPGLLAK.A R.FILIGSGNPEEGE LRPQLLDR.F R.SLVDLLPEIK.V	3334150
63		Phosphoglycerate kinase [*Arabidopsis thaliana*]	29%	47 (>46)	K.SVGDLTSADLK. G R.ADLNVPLDDNQ TITDDTR.I R.AAIPTIK.Y K.YLIENGAK.V K.FSLAPLVPR.L R.FYKEEEKNDPEF AK.K K.ELDYLVGAVSN PK.R K.RPFAAIVGGSK. V K.IGVIESLLEK.C K.FAAGTEAIANK. L K.GVTTIIGGGDSV AAVEK.V	15230595

[α]Expression ratios for each spot at five different time points (from left to right 6, 12, 24, 48 and 72 h post-challenge with the pathogen).

* Indicates statistically significant expression ratio (ER) in response to pathogen challenge at a given time point. Letters over bars were generated by Duncan's multiple-range test and time points sharing at least one alphabetical letter do not exhibit statistically significant difference in ER.

[β]Percent sequence coverage.

[γ]Mascot score for the most significant hit calculated as described in the Materials and Methods section and expressed as score/threshold value.

[δ]Sequence of the peptide(s) that were matched based on the MS/MS fragmentation patterns.

[ε]GI numbers for the proteins identified by the Mascot search.

4.3.4. Antioxidant enzymes, cellular detoxification and reinforcement of cell wall

The second largest group of proteins affected in the resistant *B. carinata* is involved in the detoxification of ROS. Production of ROS in a cell increases as a response to biotic and abiotic stresses and, because of their reactivity, poses significant harm to various cellular components (Mittler *et al.*, 2004). The detoxification of ROS is mediated by cellular antioxidant systems and many proteins with known antioxidant functions were identified in *B. carinata* as a response to *L. maculans*. For example, a protein identified as type II peroxiredoxin (Prx; Spot# 2; Fig. 4-3A; Table 4-2) increased ~4-fold in response to the pathogen very early (6h) in this resistant species while another protein which was also identified as the same type II peroxiredoxin was observed to decrease 4-fold in response to the pathogen at a later time point (48 h; Spot# 30; Fig. 4-3A; Table 4-2) in the same species. In addition, a 2-Cys Prx (Spot# 16; Fig. 4-3A; Table 4-2) was also observed to increase ~2.5-fold in response to the pathogen at 24 h. Other proteins with antioxidant functions identified in this species included dehydroascorbate reductase (Spot# 20; Fig. 4-3A; Table 4-2) and ascorbate peroxidase (APX; Spots# 22 and 24; Fig. 4-3A; Table 4-2) all of which exhibited a pathogen-induced increase at 24 hours following pathogen challenge. Our proteome-level observations suggest that in *B. carinata,* which is resistant to *L. maculans*, an increase in proteins with antioxidant activity occurs early in the interaction between the plant and pathogen whereas such an increase was not observed in the susceptible *B. napus*. In addition, the increase in enzymes from the ascorbate pathway suggests an

important role for these enzymes in dealing with the pathogen-induced oxidative stress in the resistant *B. carinata*.

Peroxiredoxins (Prxs) reduce hydrogen peroxide (H_2O_2) and alkyl hydroperoxides to water and alcohol, and these enzymes are grouped in four classes, which are (i) 1-cys Prx (ii) 2-cys Prx (iii) Type II Prx and (iv) Prx Q (Dietz, 2003). Our proteome-level analysis revealed higher levels of 2-Cys Prx and type II Prx at different time-points in the resistant species. Prxs play dual roles in plant stress responses by detoxifying ROS and by balancing signaling cascades involving ROS (Dietz, 2003). Numerous studies reported in the literature describe the importance of peroxiredoxins in ameliorating pathogen-induced oxidative stress and, in many cases, have been reported to be crucial for minimizing damage to photosynthetic machinery and increasing tolerance to pathogens (Kiba *et al.*, 2005; Baier and Dietz, 1999; Rouhier and Jacquot, 2005; Rouhier *et al.*, 2004a; Rouhier *et al.*, 2004b). Prxs are also intricately related to ascorbate-mediated detoxification of H_2O_2. For instance, transgenic *Arabidopsis thaliana* with antisense suppression of 2-Cys Prx was found to have a more oxidized ascorbate pool suggesting a partial shift of the burden from Prx- to the ascorbate-dependent pathway, especially ascorbate peroxidase (APX) which detoxifies H_2O_2 using ascorbate as a substrate (Baier *et al.*, 2000; Noctor and Foyer, 1998). As indicated earlier, spots identified as dehydroascorbate reductase and ascorbate peroxidase were found to be increased in response to *L. maculans* in the resistant *B. carinata*. An increase in APX, dehydroascorbate reductase and

peroxiredoxins has been implicated in mediating the tolerance of *B. napus* species to oxidative stress as well as pathogen attack (Sharma *et al.*, 2007).

Another protein spot exhibiting a significant increase only in the resistant *B. carinata* was identified as a putative glyoxalase I in *B. carinata* following *L. maculans* infection at the early phase of infection (6 h; Spot# 7; Fig. 4-3A; Table 4-2). The glyoxalase system is composed of two main ubiquitous enzymes: glyoxalase I and glyoxalase II, responsible for the detoxification of methylglyoxal by converting it into glutathione and lactate. Methylglyoxal is produced by the degradation of triosephosphates and threonine (Thornally, 1993) and increases during stress. A cell producing increased levels of glyoxalase could convert the methylglyoxal that accumulates in response to pathogen-induced stress into a non-toxic form, consequently protecting the cell from cytotoxic and mutagenic effects. In fact, enhanced tolerance to abiotic stress such as salinity has been observed in transgenic tobacco where the glyoxalase pathway has been enhanced through genetic engineering (Singla-Pareek *et al.*, 2003). Moreover, a role for glyoxalases in mediating plant responses to pathogens is supported by the differential expression of glyoxalase I that was also observed under conditions of osmotic stress (Zang and Komatsu, 2007) as well as in the *F. graminearum*-wheat pathosystem (Zhou *et al.*, 2006) using a proteomics-based approach.

Contrary to our observations with the resistant *B. carinata*, only one protein with presumed antioxidant activity was identified from the susceptible *B. napus*. This was a germin-like protein (Spot# 40; Fig. 4-3A; Table 4-2) which, in this species, was

observed to be decreased at 24 h following challenge with *L. maculans*. Germin-like proteins (GLPs) are a group of proteins sharing 30-70% homology to germins and were first characterized in cereals in 1980 (Bernier and Berna, 2001). These are members of cupin superfamily with a conserved 3D structure forming a six stranded ß-barrel as a common feature in all these proteins (Dunwell *et al.*, 2004). Several reports suggested a role for these proteins in strengthening the cell-wall by preventing cell expansion and signaling. These proteins were found to possess oxalate oxidase or superoxide dismutase activity, leading to the production of hydrogen peroxide, which acts as a signaling molecule in the plant defense responses to pathogens (Yamahara *et al.*, 1999; Christensen *et al.*, 2004). H_2O_2 plays a crucial role in peroxidase-catalyzed cross-linking reactions during lignification, which reinforces cell walls (Olson and Varner, 1993; Thordal-Christensen *et al.*, 1997). Constitutive expression of the wheat germin-like oxalate oxidase gene in transgenic hybrid poplar plants resulted in enhanced tolerance to the fungal pathogen *Septoria musiva* as compared to non-transgenic plants (Liang *et al.*, 2001). Additionally, gene silencing of *Nicotiana attenuata* germin-like protein (NaGLP) using virus-induced gene-silencing (VIGS) enhanced susceptibility to the insect herbivore of *N. attenuata* such as *Manduca sexta* and *Tupiocoris notatus* (Lou and Baldwin, 2006), further supporting their roles in plant defense. In our current study we observed a decrease in the intensity of one spot, identified as GLP, in the susceptible *B. napus* following *L. maculans* infection. The significant decrease in levels of GLP during this compatible interaction might lead to a decrease in H_2O_2 levels. This may result in a reduced lignification and reinforcement of plant cell walls in this species, and as mentioned earlier, we did not observe any

lignification in this susceptible species using histochemical methods. In contrast in the resistant *B. carinata*, the corresponding GLP spots did not show any decrease in intensity, which is in agreement with our histological observations indicating increased lignification in this species.

In addition to the enhanced lignification of the cell walls, we observed another protein, glycine rich protein (GRP; Spot# 1; Fig. 4-3A; Table 4-2) to increase in abundance very early (6 h) in the resistant *B. carinata* in response to the pathogen. GRPs are among the components of the plant's cell wall, tightly bound with cell wall polysaccharides and are also involved in cell wall repair (Ringlia *et al.*, 2001). Thus, an increase in GRP may lead to the reinforcement of cell walls around the site of wounding in the resistant species. Our proteome-level results suggests an overall increase in antioxidant enzymes, enhanced lignification and strengthening of the cell wall in the resistant *B. carinata*, which is not observed in the susceptible *B. napus* and these differences may be important in the eventual outcome of the interaction (i.e. tolerance or susceptibility to the pathogen).

4.3.5. Correlation between transcript and protein abundance

We were interested in comparing the protein and transcript expression of enzymes with antioxidant activities that were observed to be differentially affected by the pathogen in the tolerant and susceptible *Brassica* species. This was achieved by performing quantitative real time PCR (q-RT-PCR) analysis and, in one case, by Western blot analysis as well. The transcripts selected for measurement by q-RT-PCR

were type-II-peroxiredoxin (Type-II-Prx), 2-cys-peroxiredoxin (2-cys-Prx), germin-like protein (GLP) and ascorbate peroxidase (APX). The primer pairs used for q-RT-PCR are shown in Table 4-1 and were designed based on nucleotide sequences available in the databases. All primer pairs produced amplification products of the expected size in both species, with the exception of the *B. carinata* homologue of the GLP gene. Results of q-RT-PCR were statistically analyzed as described in the materials and methods section.

As mentioned earlier, type-II-Prx and 2-cys-Prx are different forms of peroxidases and, consistent with our proteome-level observations, the abundance of transcripts for these 2 genes were higher at 6 h in *L. maculans* challenged-resistant *B. carinata* compared to the susceptible *B. napus* (Fig. 4-6). Furthermore, at 12 and 24 h post pathogen-challenge, we observed that the transcript abundance for type-II-Prx was lower in pathogen-challenged *B. napus* compared to *B. carinata*. Thus, it is evident from the results presented in Figure 4-6 that transcript-level changes support the differential expression of type-II-Prx in these two species following pathogen-challenge. With respect to 2-cys-Prx, we did not observe any correlation between transcript abundance at 24 h post pathogen-challenge (Fig. 4-6B), at which time we observed a significant increase in protein levels in the tolerant *B. carinata* (Table 4-2). However, we did observe a significant decrease in transcript abundance for 2-cys-Prx in the susceptible *B. napus* at 12 h following pathogen-challenge, but no such decrease in transcript abundance was observed in the tolerant *B. carinata* (Fig. 4-6B). It is clear from our proteome- and transcript-level observations that these two peroxiredoxins are differentially expressed in the two genotypes in response to the pathogen.

The abundance of the transcript for GLP (Fig. 4-6D) was also observed to decrease significantly ($p<0.05$) at 12 h in blackleg-susceptible *B. napus*, although the decrease in protein abundance was observed at the later time point of 24 h (Table 4-2). Because our primers did not amplify a GLP-specific sequence in *B. carinata*, we were unable to check the transcript abundance for this gene. However, it is clear that the decline in GLP transcript abundance in the susceptible *B. napus* precedes the decline in protein levels confirming a pathogen-induced decrease in GLP in this species (Table 4-2). In the case of APX, our q-RT-PCR results did not reveal any pathogen-induced changes in transcript abundance in either species (Fig. 4-6C). Gene expression analysis is often used as a complementary approach to proteome-level analysis in order to gain a more thorough understanding of the regulation of gene expression i.e. whether the regulation is pre-or post-transcriptional. Although there have been reports of non-correlation between the transcriptome and proteome data (Yan *et al.*, 2007), our q-RT-PCR results generally correlate well with our proteome-level observations for peroxiredoxins and GLP indicating a pre-transcriptional control of gene expression.

We also wanted to confirm our proteome and q-RT-PCR results for germin like protein (GLP) by Western bloting. Polyclonal antibodies for GLP from *A. thaliana* were available and obtained from the source described in the materials and methods section. These antibodies were used in Western blots of two-dimensionally separated protein extracts from *L. maculans*-challenged *B. napus* and *B. carinata* leaf protein extracts at 24 h following challenge. The immunoreactive spots were quantitated by densitometry as described in the experimental section and a decrease in

the density of the spot reacting with these GLP-specific antibodies was observed in the susceptible *B. napus* inoculated with *L. maculans* as compared to un-inoculated control (Fig. 4-7). On the other hand, no significant difference in spot density was observed in the tolerant *B. carinata* in response to the pathogen. Apart from validating our MS identification of this protein as GLP, these Western blot results also confirm our 2-DE gel results where a significant decrease in the abundance of spots identified as GLP in the susceptible *B. napus* was observed.

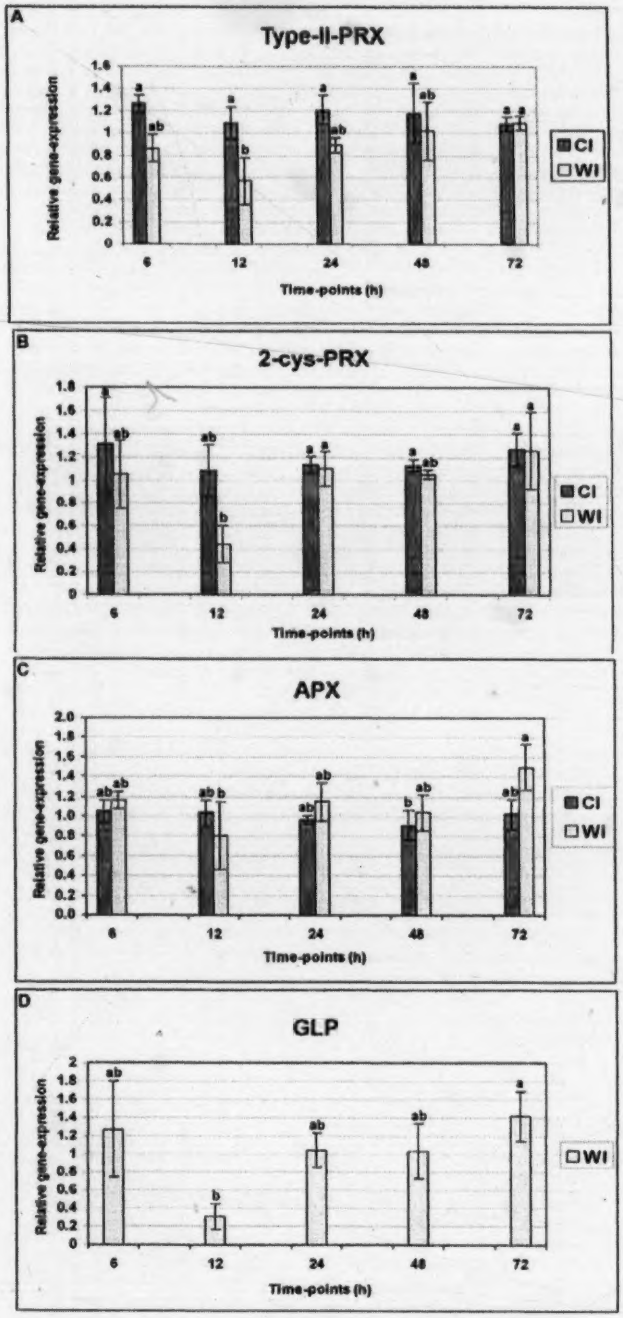


Figure 4-6. Changes in the relative abundance of transcripts of (A) Type –II PRX, (B) 2-cys PRX, (C) ascorbate peroxidase and (D) germin-like protein.

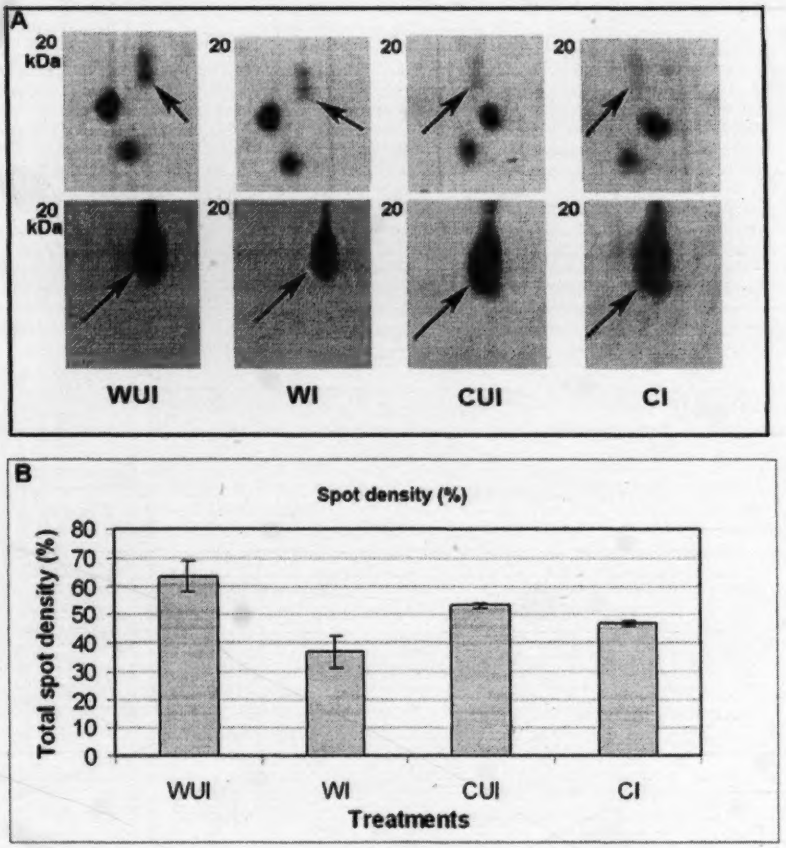


Figure 4-7. Western blot results: The top portion of panel (A) shows images of 2-DE gels used for western blot and bottom portion of panel (A) shows western blot images of *B. napus* uninoclated (WUI), *B. napus* pathogen-inoculated (WI), *B. carinata* uninoculated (CUI) and *B. carinata* pathogen-inoculated (CI). Arrows pointed towards the protein of interest. Panel (B) shows total spot density (%) of western blot of *B. napus* un-inoclated (WUI), *B. napus* pathogen-inoculated (WI), *B. carinata* un-inoculated (CUI) and *B. carinata* pathogen-inoculated (CI) respectively.

4.4. Concluding remarks

In this study, the molecular responses to *L. maculans* were investigated at the protein level in a blackleg-susceptible and -resistant *Brassica* species. Proteome-level analysis revealed the modulation of the abundance of a number of proteins involved in photosynthesis, carbohydrate metabolism, generation and detoxification of reactive oxygen species, protein folding and nitrogen metabolism in both blackleg-resistant and -susceptible species. Our results suggest that the tolerant *B. carinata* generally responds to pathogen challenge with an increase in the level of proteins with antioxidant activities whereas the susceptible species does not show such an increase. In fact, in the case of the susceptible *B. napus*, GLP levels decline upon pathogen challenge as supported by our 2-DE, q-RT-PCR and Western blot results. Furthermore, our histological observations indicate that there is enhanced lignification around the wounding site in the tolerant *B. carinata* compared to the susceptible *B. napus*. Taken together our observations suggest that the enhanced antioxidant protein levels in *B. carinata* may be at least in part responsible for mediating tolerance to this pathogen via increased lignification in addition to detoxification of ROS. The utility of transgenic approaches using genes encoding peroxiredoxins and GLP to engineer resistance to blackleg is currently being investigated in our laboratory.

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5. Cytokinin inhibits the growth of *Leptosphaeria maculans* and *Alternaria brassicae*

5.1. Introduction

Senescence is a process which involves a progressive change in a plant's physiological status and can affect the sensitivity of plants to pathogens. For example, several necrotrophic fungi such as *Alternaria alternata* (Stavely and Slana, 1971), *A. brassicae* (Conn *et al.*, 1990) and *Botrytis cinerea* (Barna and Gyorgyi, 1992) have been known to infect senescing plants due to increased susceptibility of the senescing tissue. Phytohormones such as cytokinins (CKs) play an essential role in sustaining the juvenility of plant tissues and have been investigated to understand the relationship between senescence and susceptibility towards several plant pathogens (Pogany *et al.*, 2004). For example, the formation of green islands, which is a characteristic feature of interaction between plants and biotrophic fungi such as rusts and mildews, is characterized by high levels of CKs, presumably aiding pathogen establishment and growth (Dekhuijen, 1976; Greene, 1980). Moreover, the exogenous application of CKs has been reported to enhance plant resistance during some viral and fungal infections (Clarke *et al.*, 1998; Dekker, 1963). However, the effects of CKs on the tolerance of plants to diseases caused by the hemibiotrophic fungus *Leptosphaeria maculans* (Desm.) Ces. & De Not. and the necrotrophic fungus *Alternaria brassicae* (Berk.) Sacc. have not been previously investigated.

L. maculans and *A. brassicae* are important pathogens that cause blackleg disease and Alternaria blackspot, respectively, in the economically important *Brassica* species, *B. napus*. Blackleg disease has been observed to occur on both winter and spring cultivars of oilseed, which are grown under a broad range of climates and using varying agricultural practices (West *et al.*, 2001). *L. maculans* colonizes the tissue initially as a biotroph but acts as a necrotroph by producing pycnidia (asexual fruiting bodies) in the dead tissue at a later stage (Hammond *et al.*, 1985; Hammond and Lewis, 1987). Alternaria blackspot disease causes chlorotic and necrotic foliar lesions infecting mostly the aerial parts of the plant (Verma and Saharan, 1994), and is responsible for substantial reduction in seed yield and oil quality. Up to 30% yield losses in canola have been reported in western Canada (Tewari and Conn, 1988). Strategies such as the application of fungicides and development of resistant cultivars have been employed to control these diseases; however, the former is not environmentally sustainable and is expensive whereas the latter has been overcome by the evolution of several new pathotypes of the pathogen (Sprague *et al.*, 2006). The development of durable resistance has therefore become important towards combating these diseases of canola.

Studies have suggested that approaches aimed at reducing senescence could be advantageous for reducing the damage caused by *A. brassicae*. For example, *A. brassicae* is known to produce a complex mixture of phytotoxins including destruxin B (Bains and Tewari, 1987), which causes chlorosis in infected plants (Ayer and Rodriguez, 1987). In addition, Dahiya *et al.* (1988) reported that *A. brassicae* synthesizes abscisic acid (ABA) which helps in accelerating senescence (Smart, 1994;

El-Antalby *et al.*, 1967; Arditti *et al.*, 1971; Rudnicki and Pieniazek, 1968). Since it is well known that CKs antagonize the effects of ABA and ethylene, and also delay senescence we investigated whether CKs could be useful in delaying the development of symptoms caused by *A. brassicae* in *Brassica*. We also investigated whether CKs would be useful for delaying symptoms caused by *L. maculans* because there are no reports in the literature investigating the effects of CKs on the *L. maculans-B. napus* pathosystem.

5.2. Materials and Methods

5.2.1. Plant material and pathogens

Brassica napus (cv. Westar) and *B. carinata* plants were used as susceptible genotypes for *Alternaria brassicae* experimentation, whereas only *B. napus* plants were used for *Leptosphaeria maculans* experiments because of the resistance of *B. carinata* to this pathogen (Rimmer and van den Berg, 1992; Sacristan and Gerdemann, 1986). Seedlings from both species were grown in plastic inserts (7.5 cm x 5 cm; one seed per insert) filled with Metro Mix® 290 (Grace Horticultural Products, Ajax, Ontario, Canada) in the greenhouse (22 °C day/18 °C night; 16 h photoperiod) for 3 weeks. Plants were fertilized once in 2 weeks with 200 ppm Peters® 20N-20P-20K and experiments were repeated in three independent biological replicates. Two different isolates of *A. brassicae* (UAMH 7476 and Ontario) and a virulent isolate (PG2; 77-33) of *L. maculans* were generously provided by Dr. J. P. Tewari,

Department of Agricultural, Food and Nutritional Science, University of Alberta and were used in all experiments.

5.2.2. Preparation of fungal-inoculum

All fungal strains were cultured at room temperature (21 ± 2 °C) on V8 juice-rose Bengal agar medium (Degenhardt *et al.*, 1974). *L. maculans* was cultured under light supplied by cool white-fluorescent tubes, with a 12 h photoperiod, for 10 days whereas *A. brassicae* isolates were cultured in the dark for 12 days.

Fungal spore suspensions were prepared in water by scraping spores and mycelia from the plates with the help of a glass-rod. This suspension was filtered through 4-layers of cheesecloth in order to remove mycelia and agar pieces. The suspension was centrifuged twice at 2000 x g for 5 min in a Sorvall GLC-2 centrifuge (Sorvall; Allied Scientific, Canada) and in order to remove media and metabolites from the preparation. The washed, pelleted spores were resuspended in water containing 0.05% Tween-20 and counted using a haemocytometer.

5.2.3. Detached leaf and whole plant experiments

Detached leaves were obtained from three-week old *B. napus* and *B. carinata* plants which were grown in the greenhouse as described previously. The petiole of the excised leaf was covered with an absorbent cotton-ball and placed in Petri dishes (100 mm; 2 leaves/ plate). 6-benzyl amino purine (BAP; 3 mL of 0, 0.2 mM, 0.5 mM

or 1.0 mM) was applied to the cotton-balls. After 4 h of BAP application, each leaf was wounded at 2 places on either side of the midrib using a sterile pipette tip and was inoculated with fungal spore suspension. A final spore concentration of 10^7 spores/mL was used in the case of *L. maculans* and 4 x 10^5 spores/mL for both *A. brassicae* isolates. We used 15 µL of *L. maculans* and 25 µL of *A. brassicae* spore-suspension in all these experiments. Mock-inoculated controls (un-inoculated) were treated with sterile distilled water containing 0.05% Tween-20 at the wounding site. Photographs were taken on the 5^{th} day post-inoculation for leaves treated with *A. brassicae* and 11^{th} day post-inoculation for leaves treated with *L. maculans*.

In the case of whole-plant experiments, three-week old plants (which were in the humidity chamber for at least 24 h prior to the experiment) were placed in the greenhouse, allowed to dry, and sprayed to drench with 25 ml of BAP (0, 0.2 mM, 0.5 mM, and 1.0 mM). They were then allowed to remain in the greenhouse for an additional 4 h. Leaves (first or second true leaves) were wounded as described above, the fungal suspension applied and the plants were returned to the humidity chamber for 24 h. They were then moved back to the greenhouse and monitored for symptoms. Photographs were taken on the 10^{th} day for *A. brassicae* and 11^{th} day post-inoculation for *L. maculans* treated plants. Disease scoring was conducted using APS Assess software (Image Analysis Software for Plant disease Quantification; The American Phytopathological Society).

5.2.4. Fungal growth on cytokinin-supplemented media

The aforementioned media for the growth of both fungal pathogens containing V8 juice-rose Bengal was used in these experiments. Three different concentrations (0.2 mM, 0.5 mM and 1.0 mM) of the cytokinins (CKs) 6-benzyl amino purine (BAP) and kinetin (Sigma-Aldrich Co. Ltd, St. Louis, MO) were prepared by dissolving in a small amount of 1N NaOH after which the final volume was adjusted with distilled water to achieve the appropriate concentration of CKs and were filter-sterilized using small filter units (25 mm, 0.22 µm pore size; Fisher Scientific, Ireland). Control plates with no CK supplementation contained distilled water with similar amounts of 1N NaOH as in the CK solutions. The pH of the CK-supplemented media was also determined in order to ensure that the addition of CK did not cause drastic changes in pH. Autoclaved media was allowed to cool to 60 ^{o}C prior to supplementing with CK solutions or water/NaOH (for controls) before pouring into 100 mm Petri dishes. Medium containing adenine was prepared by dissolving in 1N HCl and incubating at 50 ^{o}C in a water-bath till completely dissolved, while adenine hemisulfate was dissolved in distilled H_2O, together with appropriate HCl controls.

Fungal agar blocks of 4 mm diameter containing both spores and mycelia were placed in the middle of the plates containing the CKs, adenine and adenine hemisulfate supplements or their respective controls. Plates with *A. brassicae* were incubated in the dark whereas those with *L. maculans* were kept under white light with a 12 h photoperiod for 15 days at room temperature to monitor fungal growth. The

diameter of fungal growth was measured on 15th day. The entire experiment was repeated at least three times.

5.2.5. Histological studies

Hormone and non-hormone-treated pathogen-inoculated leaves as well as un-inoculated (mock-infected) controls were cut into small (5 mm x 10 mm) pieces, and fixed in FAA (formalin, acetic acid and ethyl alcohol; Yeung and Saxena, 2005) under vacuum at room temperature overnight. Following fixation, they were subsequently dehydrated in a series of graded ethanol/ water solutions, changed to toluene and later infiltrated with Paraplast® using a Fisher Histomatic Tissue Processor (Model 166; Pittsburgh, PA, USA). Paradermal sections (7 µm thickness) were prepared using an AO Rotary microtome (Spencer 820; Buffalo, NY, USA), affixed to the glass slides, de-paraffinated with toluene, rehydrated to 50% ethanol and stained with Aniline blue in Lacto-phenol for 10 min (Larone, 1995). After this, stained sections were rinsed with water (3 x 3 min each) and counterstained with acidified Eosin Y for 1 min (Dougherty, 1981), dehydrated in ethanol followed by toluene and mounted with DPX® (Electron Microscopy Sciences, Hatfield, PA, USA) mounting medium. The sections were viewed with a Leica DM *RXA* microscope (Leica Microsystems, Wetzlar, Germany), analyzed using Macrofire™ software (Optronics, Goleta, CA, USA) and photographed with an Optronics digital camera (Optronics, Goleta, CA, USA).

5.2.6. Statistical analysis

Statistical analysis of disease severity results was performed using analysis of variance (ANOVA) with the mixed model procedure of SAS version 9.1 (Statistical Analysis system; SAS Institute Inc., Cary, NC, USA).

5.3. Results

5.3.1. Effects of CKs on plant-pathogen interaction

The effects of the CK i.e. BAP, were investigated using both detached leaf and whole plant systems. When detached leaves were treated with various concentrations of BAP and challenged with the pathogen, a significant reduction in the lesion caused by *L. maculans* infection in the blackleg susceptible *B. napus* was observed at all concentrations when compared to the untreated controls (Fig. 5-1A). Although a statistically significant effect on disease severity was not observed (Fig. 5-1A), with increasing concentration of BAP, but reduction in lesion size were observed on those leaves treated with higher concentrations of BAP (Fig. 5-1A).

Similarly, BAP-treated *B. napus* leaves infected with both *A. brassicae* isolates (UAMH 7476 and Ontario isolates) also demonstrated a reduction of visual symptoms (Fig. 5-1B) and a significant ($p<0.05$) reduction in severity (Figs. 5-1B and C). No significant effects of concentration of BAP used were observed on severity of disease caused by this pathogen on *B. napus* leaves (Figs. 5-1B and C). Because of the fact

that *B. carinata* has been reported to exhibit a degree of tolerance to *A. brassicae* (Bansal *et al.*, 1990), we wanted to investigate whether BAP treatment could further increase the tolerance of this species to both isolates of *A. brassicae*. The results from detached *B. carinata* leaf experiments are shown in Figure 5-1 C. As reported in the literature (Bansal *et al.*, 1990), *B. carinata* was observed to be more tolerant to *A. brassicae* when compared to *B. napus*; however, the application of BAP did not significantly affect disease symptoms or severity (Figs. 5-1B, C & 5-2B, C). The effects of BAP on the interaction of *L. maculans* with *B. carinata* were not investigated because of the resistance of this species to that pathogen (Rimmer and Van den Berg, 1992; Sacristan and Gerdemann, 1986).

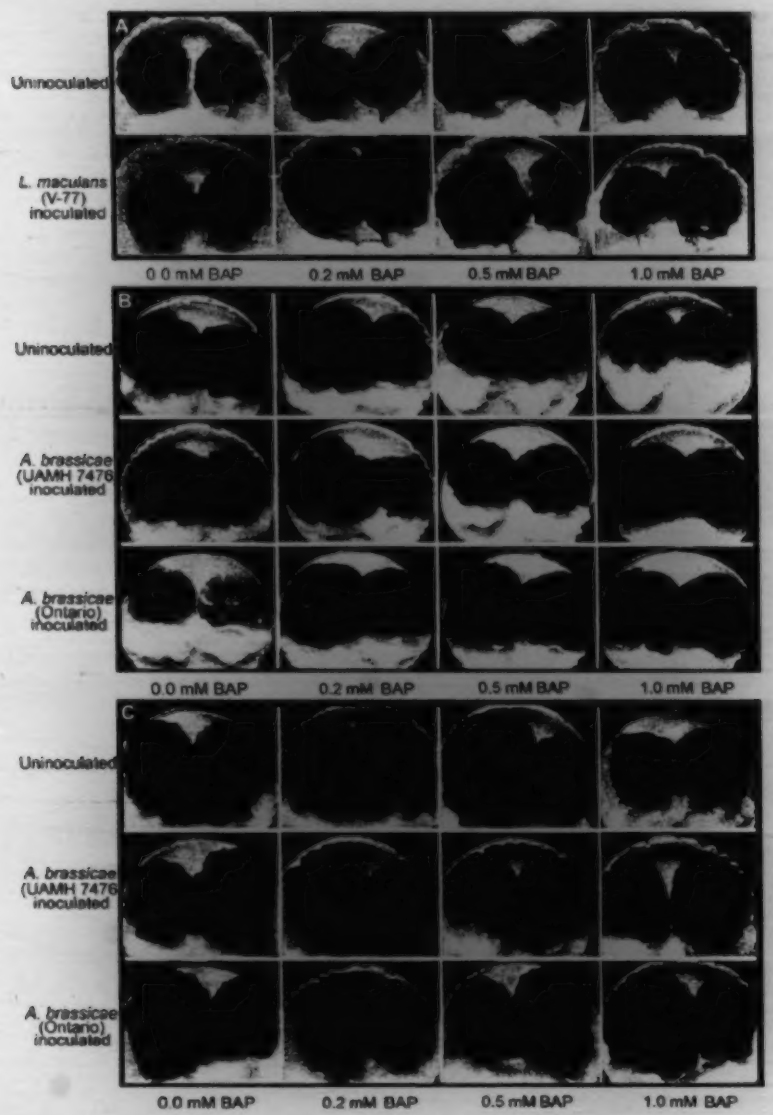

Figure 5-1. Effects of BAP on the interaction of pathogens with detached leaves of *Brassica.* (A) symptoms elicited on *B. napus* (cv. Westar) leaves by *L. maculans* (B) by *A. brassicae* isolates on *B. napus* and (C) by *A. brassicae* isolates on *B. carinata.*

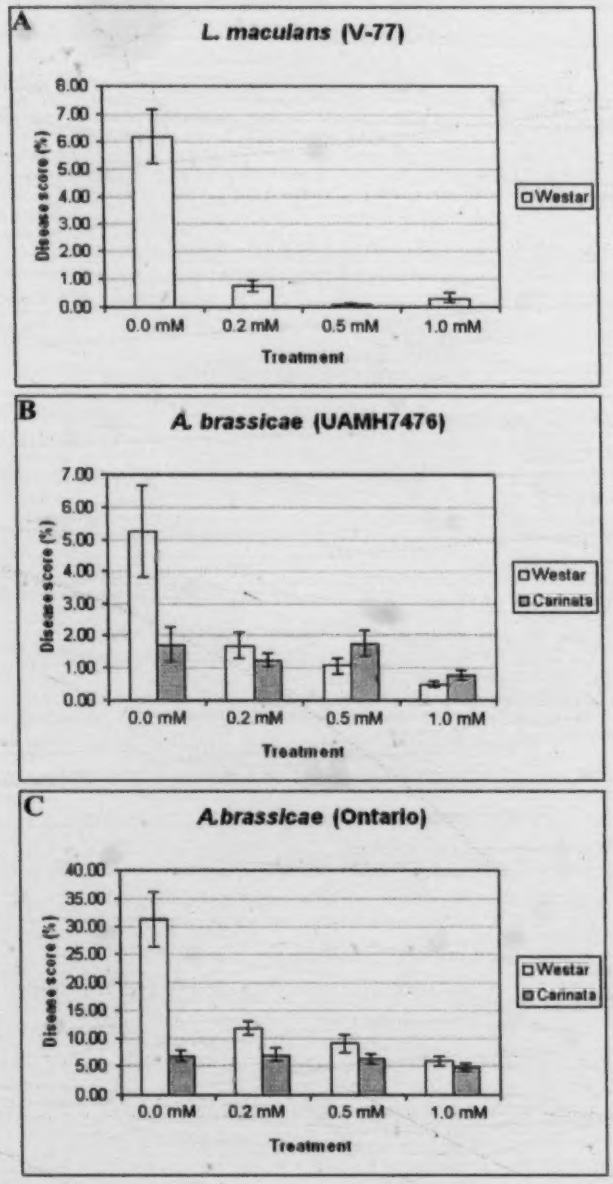

Figure 5-2. Disease symptoms induced by *L. maculans* and *A. brassicae* isolates on *Brassica* plants in a detached leaf experiment. Effects of various concentrations of BAP treatments on the disease severity score (%) induced by *L. maculans* on *B. napus* (A), by *A. brassicae* isolate UAMH 7476 (B) and Ontario (C) on *B. napus* and *B. carinata*.

In the whole plant experiments, a similar, significant reduction in the disease symptoms caused by *L. maculans* was found in BAP-sprayed *B. napus* plants when compared to their control counterparts (Fig. 5-3A). In addition, high concentrations of BAP (i.e. 0.5 mM and 1.0 mM) showed significant disease reduction as compared to the lower hormone concentration of 0.2 mM. In the case of *A. brassicae*, we tested only the Ontario isolate in these whole-plant experiments because this isolate was more virulent than other one (UAMH 7476). Our observations confirmed once again that significant differences in disease symptom development in both susceptible *B. napus* and moderately tolerant *B. carinata* but no significant differences within three different concentrations of BAP tested (Fig. 5-3B). In summary, our detached leaf and whole plant experiments indicated that a significant degree of protection against both *L. maculans* and *A. brassicae* could be provided by BAP treatment. Furthermore, due to the inability of BAP treatment to further increase the tolerance in the more tolerant *B. carinata*, it was hypothesized that BAP may be exerting its effects by directly inhibiting fungal growth as opposed to altering plant defense responses. This hypothesis was tested by supplementing BAP, kinetin and other CKs on fungal growth media and assessing the effects of these supplements on fungal growth *in vitro*.

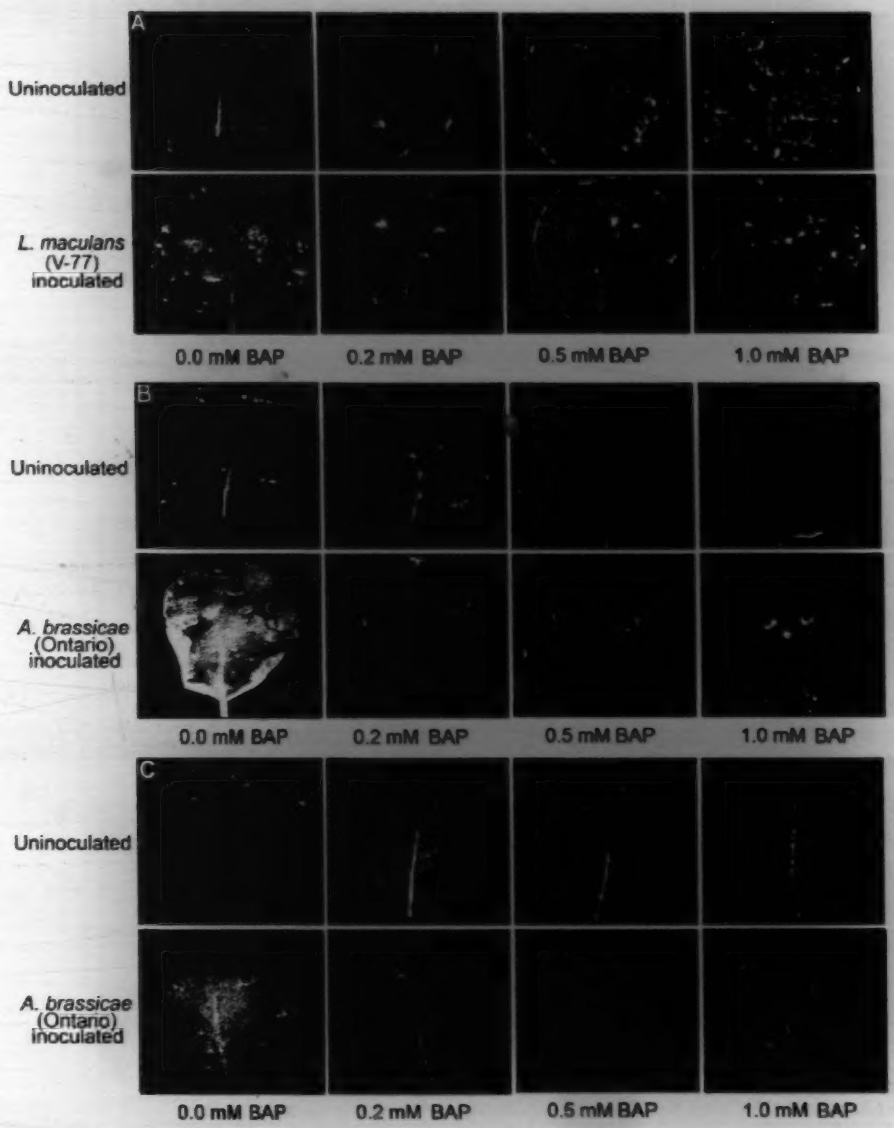

Figure 5-3. Effects of BAP on symptoms elicited by *L. maculans* and *A. brassicae* on *Brassica* in a whole-plant experiment. *B. napus* (cv. Westar) plants challenged with (A) *L. maculans*; (B) *A. brassicae* (Ontario isolate) and (C) *B. carinata* plants challenged with *A. brassicae* (Ontario isolate).

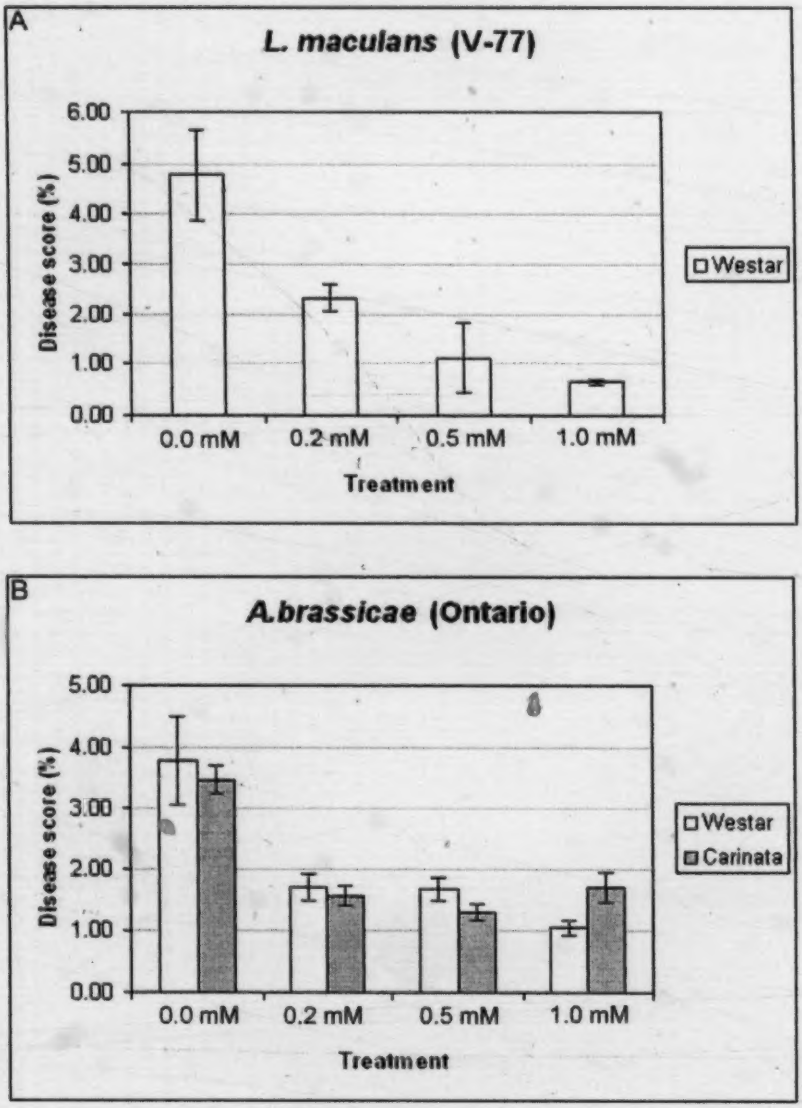

Figure 5-4. Severity of disease symptoms induced by *L. maculans* and *A. brassicae* (Ontario isolate) on *Brassica* plants in a whole-plant experiment. Effects of various concentrations of BAP treatments on the disease severity score (%) induced by *L. maculans* on *B. napus* (A) and by *A. brassicae* on *B. napus* and *B. carinata* (B).

5.3.2. Effect of CKs on fungal growth

In order to test the effect of CKs on fungal growth in Petri dishes, fungal growth media containing CKs were prepared as described earlier. The pH of each medium was measured to ensure that no significant changes occurred as a result of CK addition. In the case of *L. maculans,* it was observed that 0.2 mM BAP had significantly ($p<0.05$) reduced fungal growth when compared to the control (Fig. 5-5 and Table 5-1). At the higher concentrations of BAP (0.5 mM and 1.0 mM), a further significant reduction of colony diameter was observed (Fig. 5-5 and Table 5-1). These results show that BAP inhibits the growth of *L. maculans in vitro*. In order to determine whether the effects were unique to BAP, we also tested the ability of kinetin to inhibit the growth of *L. maculans in vitro*. We observed that kinetin was not able to significantly ($p<0.05$) reduce fungal growth (Fig. 5-6 and Table 5-1) when compared to the controls.

When the ability of BAP and kinetin to inhibit the growth of both isolates of *A. brassicae* was investigated, we observed that both CKs could significantly ($P<0.05$) reduce fungal colony diameter in a dose-dependent manner (Figs. 5-5, 5-6 and Table 5-1). Compared with the effects of CKs on *L. maculans*, BAP supplementation of the growth media led to a more drastic growth inhibition of *A. brassicae* in these experiments with no growth being observed at the highest concentration (1.0 mM) of BAP tested (Fig. 5-5 and Table 5-1). In addition, kinetin was also able to inhibit the growth of both isolates of *A. brassicae* to a greater degree compared to *L. maculans* (Fig. 5-6 and Table 5-1).

Since one of the differences between BAP and kinetin is the presence or absence of a benzene ring, we hypothesized that the benzene ring in BAP was probably responsible for the higher fungicidal/fungistatic activity of BAP. We therefore tested the ability of adenine and adenine hemisulfate (known to have CK-like activity; Chandra *et al.,* 2003) for their ability to inhibit fungal growth in Petri dishes. Our results (Figs. 5-7, 5-8 and Table 5-1) indicated that neither compounds were able to significantly affect the growth of either pathogen supporting our suggestion that the benzene ring structure in BAP may be responsible for the higher fungicidal/fungistatic activity. Therefore, it appears that CKs BAP and kinetin significantly inhibit the growth of two canola pathogens *L. maculans* and *A. brassicae* with the inhibitory activity being higher against *A. brassicae* compared to *L. maculans*. Furthermore, it appears that BAP is better of the two CKs when it comes to inhibition which may, in part, be due to the presence of a benzene ring in its structure.

Table 5-1: Effect of CKs and other additives on pathogen growth on Petri dishes.

	Colony Diameter (mm)		
	L. maculans	***A. brassicae* (UAMH7476)**	***A. brassicae* (Ontario)**
No hormone	60.3 ± 1.85	65.3 ± 0.67	42.7 ± 1.67
BAP (0.2 mM)	57.7 ± 0.88	27.0 ± 1.00	19.3 ± 1.67
BAP (0.5 mM)	53.7 ± 0.33	1.00 ± 0.00	1.00 ± 0.00
BAP (1. 0 mM)	51.3 ± 0.33	No growth	No growth
Kinetin (0.2 mM)	63.3 ± 0.67	49.3 ± 0.88	32.7 ± 3.33
Kinetin (0.5 mM)	64.0 ± 1.15	33.3 ± 1.45	24.3 ± 3.33
Kinetin (1.0 mM)	62.7 ± 0.67	21.0 ± 0.00	12.7 ± 1.67
No chemicals	66.7 ± 0.67	63.0 ± 1.15	36.0 ± 1.73
Adenine (0.2 mM)	71.0 ± 0.00	65.3 ± 0.67	37.0 ± 2.08
Adenine (0.5 mM)	71.0 ± 0.00	63.0 ± 1.00	35.0 ± 1.00
Adenine (1.0 mM)	70.7 ± 0.33	65.7 ± 0.67	35.7 ± 0.33
AHS (0.2 mM)	71.0 ± 0.00	63.7 ± 0.33	34.7 ± 1.33
AHS (0.5 mM)	70.3 ± 0.67	62.7 ± 1.20	35.0 ± 1.00
AHS (1.0 mM)	70.3 ± 0.67	64.0 ± 1.00	34.7 ± 1.33

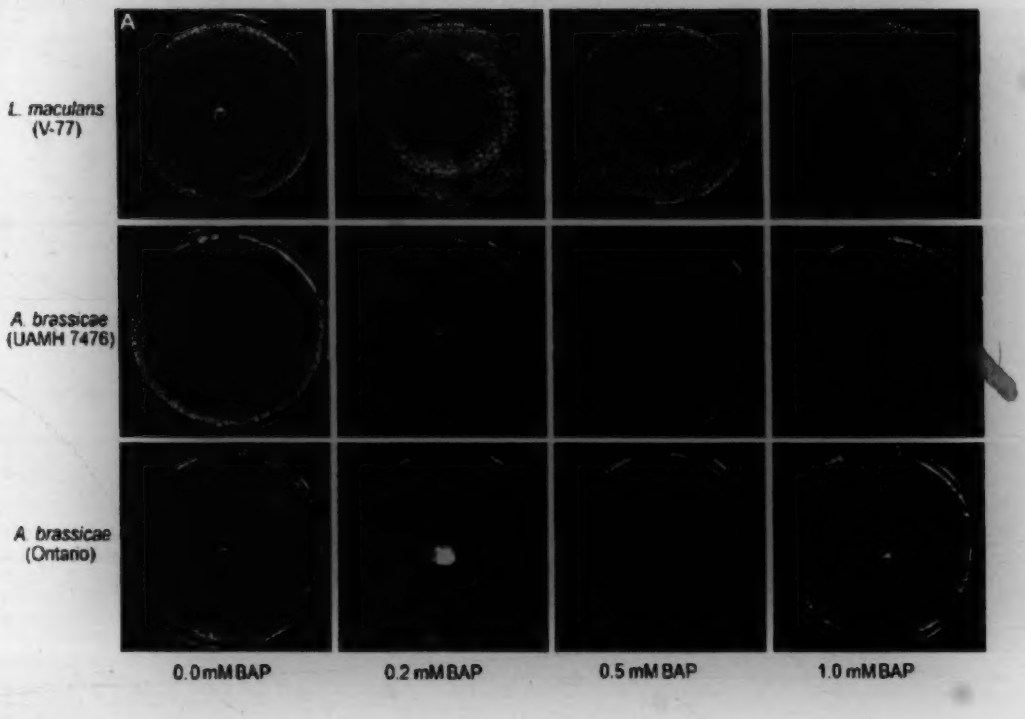

Figure 5-5. Effects of BAP on the growth of *L. maculans* and *A. brassicae* isolates. Column (A) illustrates growth on media containing various concentrations of BAP.

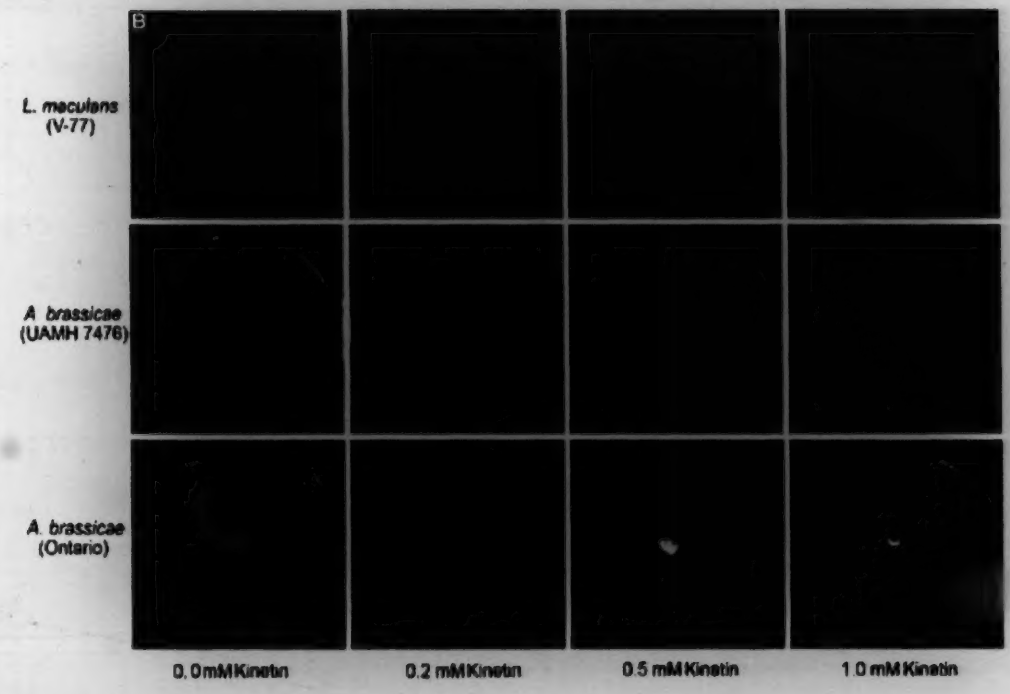

Figure 5-6. Effects of kinetin on the growth of *L. maculans* and *A. brassicae* isolates. Column (B) illustrates growth on media containing various concentrations of kinetin.

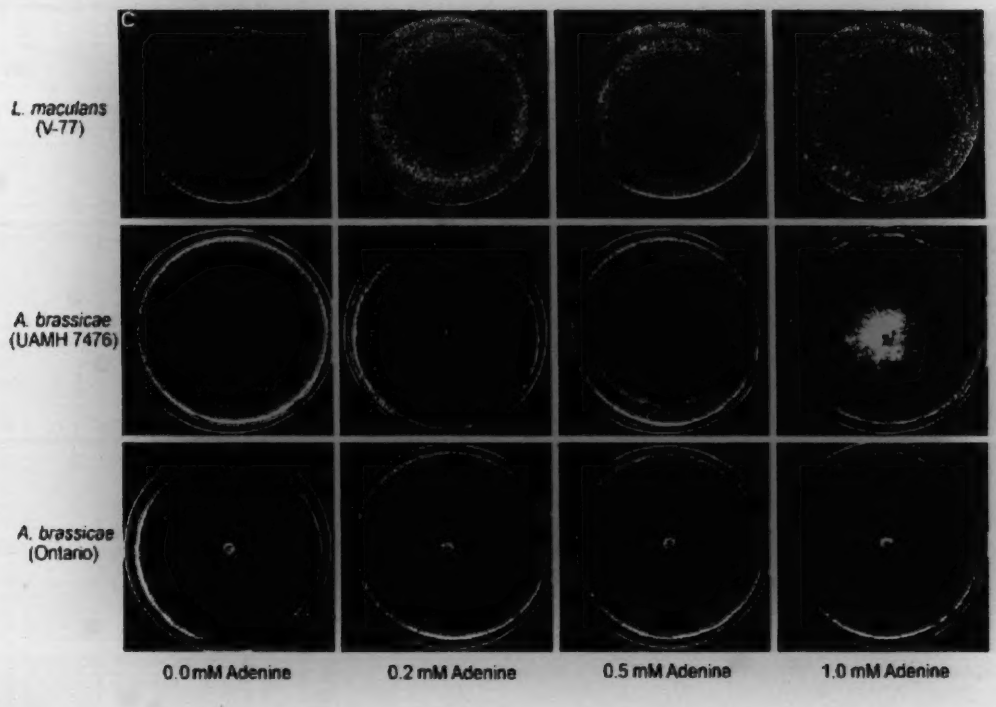

Figure 5-7. Effects of adenine on the growth of *L. maculans* and *A. brassicae* isolates. Column (C) illustrates growth on media containing various concentrations of adenine.

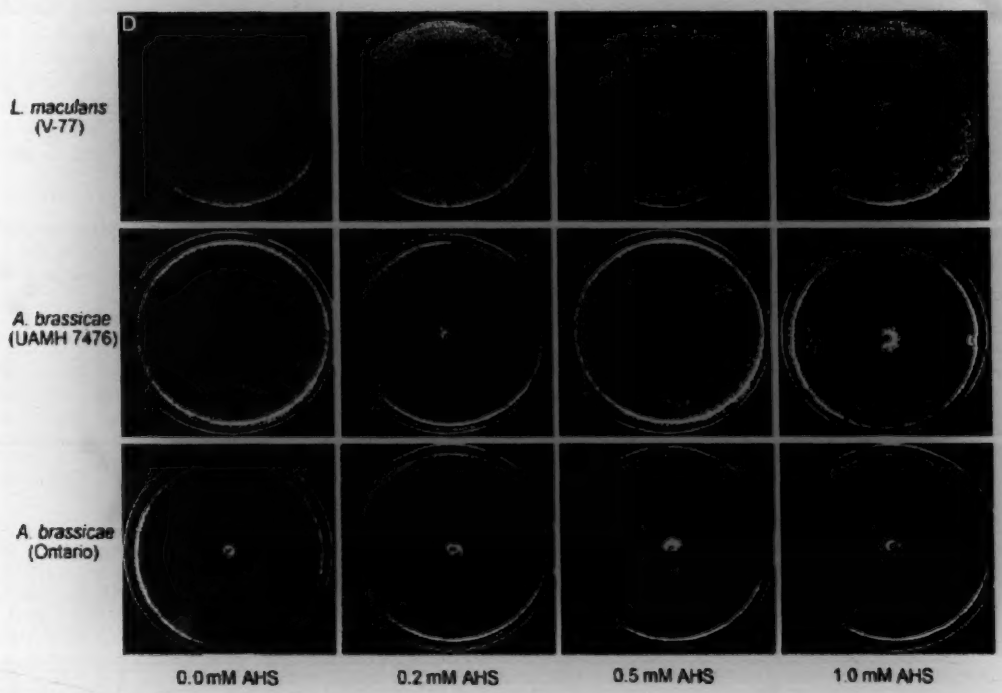

Figure 5-8. Effects of adenine hemisulfate on the growth of *L. maculans* and *A. brassicae* isolates. Column (D) illustrates growth on media containing various concentrations of adenine hemisulfate.

5.3.3. Histological characterization

The effects of BAP treatment on the interaction of the two pathogens with *Brassica* was investigated using light microscopy. Mock-inoculated controls revealed dead and injured cells at the wounding site in both species (Fig. 5-9). In the case of untreated *B. napus* inoculated with *L. maculans* the presence of dead and lysed cells could be observed with extensive fungal mycelial growth and the presence of pycnidia similar to previous reports in the literature (Fig. 5-9; Li et *al.*, 2004). Similar results were observed in 0.2 mM BAP-treated *B. napus* inoculated with *L. maculans,* whereas a reduction in mycelial growth and the absence of pycnidial bodies could be observed at 0.5 mM BAP and no fungal growth could be seen at 1.0 mM BAP (Fig.5-9).

In the case of *A. brassicae* the fungal mycelial growth could be observed in both hormone-treated and untreated samples although the mycelial growth was reduced at the highest concentration (1.0 mM BAP) in both species of *Brassica*. Furthermore, the reduction in fungal growth was more evident in *B. carinata* as compared to *B. napus* at this concentration. Our histological observations correlate well with phenotypic observation of hormone-treated and untreated *Brassica* species-infected with different fungi.

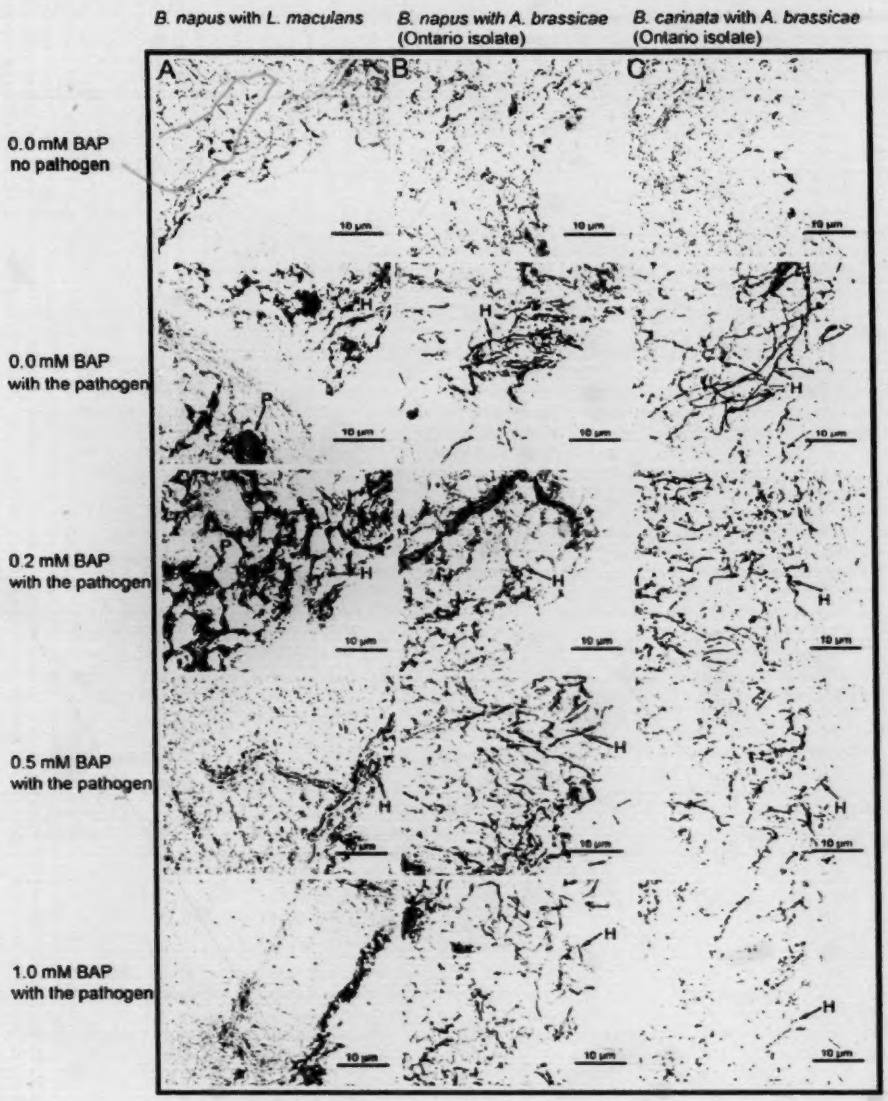

Figure 5-9. Histological studies of the responses of hormone-treated and untreated leaves. Panels in column A illustrates the effects of various concentrations of BAP on the interaction of *B. napus* (cv. Westar) leaves with *L. maculans*; with *A. brassicae* (Ontario isolate), column B and *B. carinata* with *A. brassicae* (Ontario isolate), column C. Arrows indicate fungal hypha (H).

5.4. Discussion

CKs such as kinetin and BAP are known to delay senescence by preventing degradation of chlorophyll and photosynthetic proteins, and are more effective than zeatin and 2ip (6-3-methyl-2-butenylaminopurine) in this regard (Yamada *et al.,* 1964; Badenoch-Jones *et al.,* 1996; Hamzi and Skoog, 1964; Tetley and Thimann, 1974). Injured and senescing plants are more susceptible to pathogen attack (Barna and Gyorgyi, 1992; Conn *et al.,* 1990) and therefore, exogenous application of CKs may aid in the delay and reduction of disease. For example, transgenic tobacco lines with higher CK levels were observed to be more tolerant to tobacco necrosis virus (TNV; Pogany *et al.,* 2004). Additionally, a combination of BAP and the auxin α-naphthaleneacetic acid (NAA) has been reported to inhibit the growth of *Phaeolus schweinitzii* (Hrib *et al.,* 1993). CK also had a suppressive effect on the wildfire disease of tobacco caused by the bacterium *Pseudomonas tabaci* (Lovrekovich and Farkas, 1963). In another study, CKs were observed to induce resistance in *Phaseolus vulgaris* to the virus white clover mosaic potexvirus (Clarke *et al.,* 1998) and it also affected the growth of the fungus *Erisiphe cichoracearum* DC on leaf discs of tobacco (Cole and Fernandes, 1970). CKs have also been reported to enhance the resistance of barley against the fungal pathogen *Erysiphe graminis* f. sp. *hordei*, whereas ABA increased the susceptibility of the host towards the pathogen (Edwards, 1983). As mentioned earlier, *A. brassicae* synthesizes ABA (Dahiya *et al.,* 1988) and CKs are well recognized to have ABA-antagonistic effect, which could counteract the effects of ABA and prevent disease induction and progression. Results from this study

clearly demonstrate that the CK BAP can reduce symptoms elicited by both *L. maculans* and *A. brassicae*. Furthermore, our results also demonstrate that BAP inhibits the *in vitro* growth of both pathogens with a higher inhibitory effect against *L. maculans* (Fig. 5-5). To the best of our knowledge, even though CKs have been implicated in other host-pathogen interactions, this is the first direct demonstration of a protective role of BAP against these two pathogens.

BAP and kinetin have also been reported to inhibit the mycelial growth and sexual reproduction such as production of oogonia in *Saprolegnia australis,* whereas adenine and hypoxanthine stimulated the oogonial production (Elliott, 1967). Interestingly, it was also observed that though both kinetin and BAP evoked similar responses to *S. australis,* BAP induced more inhibition of growth and sexual reproduction at a lower concentration as compared to kinetin (Elliott, 1967). Our histological observations also indicate that higher concentrations of BAP drastically reduce mycelial growth in the case of *L. maculans* and also inhibited the formation of pycnidia. The reduction in pycnidia formation at 0.5 mM BAP and their absence at higher concentrations could be analogous to the reduction of oogonia production in *Saprolegnia* due to cytokinin treatment (Elliott, 1967). Thus, a reduction in pycnidia formation by BAP treatment may aid in the reduction of secondary infection within the plant tissue.

We also observed that BAP was better at inhibiting fungal growth when compared to kinetin, which may be due to the structural differences between the two types of CKs used in these studies. The presence of the benzyl group in BAP seems to be responsible for the better activity of this compound compared to kinetin.

Compounds containing benzyl group in their structures have been reported to be inhibitory to fungal growth; for example, cinnamic acid reduced the growth of *Neurospora crassa* by approximately 94% within 24 h incubation (Neves *et al.*, 2005, Said *et al.*, 2004). This theory needs to be investigated further through the synthesis of appropriate compounds and testing their fungistatic/fungicidal activity.

In conclusion, our results indicate that BAP has an inhibitory effect towards two important canola pathogens. This effect seems to be more pronounced in the case of the hemibiotrophic fungus *L. maculans* when the effects were investigated using detached leaves or whole plants. However, the inhibitory effects of BAP on the necrotrophic fungus *A. brassicae* are higher in the Petri dish experiments compared to *L. maculans*. These results suggest that, while BAP has direct inhibitory effects on the growth of the pathogen *in vivo* and *in vitro*, there will be additional effects on the plant which would also affect pathogen establishment and growth. Indeed, it is well known from reports in the literature that CKs activate numerous plant defense response genes (Schafer *et al.*, 2000). Our efforts are now directed towards understanding the roles of specific CK-inducible defense genes in relation to these two pathosystems.

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6. General discussion

Continuous improvement in plant productivity has always been at the forefront of man's quest to provide an adequate supply of nutritious food for the global population. Before the advent of the scientific approach to enhance plant production, efforts were made to select for crops with beneficial agronomic characteristics (Smith, 1998). Rapid advances in the field of plant breeding resulted in the development of high-yielding and disease-resistant crop varieties along with the increasing usage of synthetic chemicals to boost plant growth and control pathogens and pests (Jacobsen, 1997). While significant strides made using the classical plant breeding approach resulted in the widely acclaimed "green revolution" (Evenson and Gollin, 2003), an equally important revolution, the "gene revolution" was developed due to the ability to manipulate, mutate and analyze discrete DNA fragments (Brooks, 1987; Carter, 1986; Moores, 1987). This coupled to the capability to transform defined gene sequences into plants (Horsch *et al.,* 1985), was soon exploited to generate transgenic crops with agronomically desirable features resulting in a gene revolution.

The steadily accumulating information on the uncharacterized genome soon assumed huge proportions leading to high-throughput techniques such as oligonucleotide and cDNA microarrays allowing novel insights into molecular mechanisms governing metabolic and regulatory pathways and complex signaling networks. However, an understanding based on the genomic and transcriptomic analyses is incomplete because although measurement of mRNA levels is a valuable molecular tool, the final activity of a gene is determined by the protein that it encodes.

Consequently, mRNA levels are not always consistent with the abundance of cognate proteins. Furthermore, expression of a single gene can generate different protein species due to post-transcriptional (i.e. alternative splicing, mRNA processing) and post-translational mechanisms (i.e. phosphorylation, acetylation, glycosylation, alkylation, ubiquitination). Additionally, protein functions are frequently dependent on their interaction with other biomolecules and environmental signals. Therefore, proteomics, defined as the systematic analyses of the proteome (the protein complement of the genome) has emerged as an important tool to offer a comprehensive understanding of the subtle molecular processes underlying biological phenomena, which may not be possible using other approaches. While proteomes of model micro-organisms like *E. coli* (Yun *et al.*, 2007), *S. cerevisiae* (Hodges *et al.*, 1998) and higher organisms such as humans (Peri *et al.*, 2003) have been well-characterized, little or no information is available for most of the plant proteomes. However, proteomics-based investigations into plant responses to stress are becoming increasingly popular (Kav *et al.*, 2007).

Generally, plants are resistant to most pathogenic organisms that they encounter, primarily because of their ability to recognize potential invading pathogens and activate defense mechanisms. However, certain pathogens cause disease in certain plant species because of their ability to avoid detection and/or overcome the host defense system. Although disease-resistant varieties have been generated using traditional and non-traditional means, pathogens continue to evolve into more virulent strains that are responsible for causing devastating crop losses, outlining the urgency to identify alternative and durable sources of disease resistance.

The interaction between the host and pathogen triggers biochemical events that set into motion signal transduction cascades, eventually leading to the activation of pathogen-responsive genes. The identification of genes expressed and the proteins synthesized during host-pathogen interaction is of immense interest as some of these proteins are directly involved in resisting the pathogen attack and conferring disease tolerance on the plant. Thus, proteome-based expression profiling is necessary to augment the information generated using transcriptome-based approaches in order to obtain a complete understanding of the regulatory elements in plant-pathogen interactions.

Canola (*Brassica napus*) is one of the major crops cultivated in Canada and is estimated to contribute more than $13 billion in economic activity to the Canadian economy (www.canola-council.org). With its low saturated fat level, excellent balance of polyunsaturated and monounsaturated fats, its versatility and light taste, canola oil has found an important place in the world's kitchens, food processing companies and restaurants. Phoma stem canker (causal agent *L. maculans*) and Alternaria blackspot (causal agent *A. brassicae*) severely limit canola crop productivity not only in Canada but also in other parts of the world such as Australia, France, India and China. We therefore decided to characterize proteome-level changes in canola following inoculation with these pathogens.

In the first study, leaves from blackspot-tolerant and -susceptible *B. napus* lines were inoculated with *A. brassicae* spore suspension. Total protein was extracted from the plant tissue at different time-points following pathogen challenge and subjected to two-dimensional gel electrophoresis (2-DE). Comparison of the protein

profiles exhibited significant changes in spot intensities of 48 proteins in the blackspot tolerant line (wherein 41 spots were up-regulated and 7 were down-regulated). In the blackspot-susceptible line, the levels of 23 proteins were notably affected (wherein 4 spots were up-regulated and 19 were down-regulated). An ESI-Q-ToF-MS/MS analysis led to the identification of 38 protein spots (out of a total of 71 that were analyzed from the two lines) and were sub-grouped into distinct functional clusters. While some of these proteins participate in general metabolism, several of the identified proteins were involved in the production of reactive oxygen species (such as germin-like protein), detoxification of reactive oxygen species (namely peroxiredoxin, glutathione peroxidase and a putative dehydroascorbate reductase) and ROS signal transduction pathway (nucleoside diphosphate kinase). Reactive oxygen species play a prominent role in both the early and later stages of plant pathogenesis, functioning directly as anti-pathogenic agents and indirectly as cellular signaling molecules for induction of local and systemic resistance. Although the increase in levels of many of the identified proteins were quite modest, we believe that the proteome profile of *B. napus* upon challenge with *A. brassicae* offers a close approximation of the processes and pathways that are exploited by the host plant in order to successfully ward off challenge posed by this pathogen.

Another study was performed to characterize the *Brassica* proteome upon inoculation with *L. maculans*, causal agent of the devastating Phoma stem canker or blackleg disease. Although blackleg-resistant canola varieties have been developed, many new isolates of this fungus that have evolved to overcome this resistance have also been identified. Limited understanding of the molecular and biochemical

processes that accompany this host-pathogen interaction has been a stumbling block for breeding blackleg resistant canola varieties. Consequently, increasing attention is being focused on the possible exploitation of germplasm sources displaying resistance to blackleg. *Brassica carinata*, a wild relative of canola, has been observed to display broad-spectrum resistance to different isolates of blackleg. We decided to profile the proteome-level changes in blackleg-resistant *B. carinata* upon inoculation with *L. maculans* and in susceptible *B. napus*. Accordingly, leaves from these two species were inoculated with spore suspension and the total protein extracted at five different time-points post-inoculation (6, 12, 24, 48, and 72 h) and subjected to 2-DE. Protein spots displaying reproducible intensity changes were excised and identified using ESI ion trap MS/MS. A total of 37 and 27 protein spots were observed to be differentially altered in *B. carinata* and *B. napus* respectively, at various time-points following pathogen challenge. Interestingly, in the case of *B. napus*, only one spot showed increased intensity whereas the remaining 26 spots displayed a reduction in their intensities. Enzymes involved in photosynthesis (RUBISCO), Calvin cycle (3-phosphoglycerate kinase, fructose bisphosphate aldolase and glyceraldehyde-3 phosphate dehydrogenase), citric acid cycle (malate dehydrogenase) and carbon metabolism (dihydrolipoyl dehydrogenase) comprised an important sub-set of proteins that were significantly down-regulated at various time-points in the susceptible *B. napus*. This suggested that most of the photosynthetic and carbon-metabolism-related enzymes were adversely affected in the susceptible-line relative to the resistant one, possibly as an unavoidable result of necrotic tissue damage following infection. Apart from these proteins, a large group of proteins identified from the tolerant species was

involved in the production and detoxification of reactive oxygen species (ROS) in addition to some implicated in plant cell wall reinforcement.

Taken together from both aforementioned studies, our findings suggest that plants tolerant to these pathogens appear to possess a large group of antioxidant enzymes along with mechanisms for cell wall reinforcement. Interestingly, in both studies, GLP, which leads to the production of H_2O_2, was the only protein observed to be down-regulated in blackleg and blackspot-susceptible Brassica genotypes suggesting a potential role for GLP in these compatible interactions.

Another study described in this thesis focused on the effect of cytokinin (particularly BAP) on the *in planta* and *in vitro* growth of *L. maculans* and *A. brassicae*. The senescing tissues of *B. napus* are highly susceptible to necrotrophic fungi such as *Botrytis cinerea* (Barna and Gyorgyi, 1992) and *A. brassicae* (Conn *et al.*, 1990). Several plant hormones such as cytokinin, abscisic acid (ABA) and ethylene are known to play an important role in regulation of sensescence. ABA and ethylene promote senescence whereas cytokinins (CKs) delay the onset of senescence (Gan and Amasino, 1997). In addition to their role in delaying senescence in plants, cytokinins play a proactive role in the reduction of stress imposed by several processes. For instance, kinetin and BAP have been reported to inhibit the mycelial growth and sexual reproduction such as production of oogonia in the oomycete *Saprolegnia australis* (Elliott, 1967). Exogenous application of cytokinin decreased the activity of lipoxygenase in *Pisum sativum* L. foliage and *Vigna unguiculata* L. and retarded leaf senescence (Grossman and Leshem, 1978; Swamy and Suguna, 1992). Cytokinins were also observed to aid in the scavenging of active oxygen caused by

ozone damage in *Phaseolus vulgaris* L. (Pauls and Thompson, 1982). Increased activities of superoxide dismutase (SOD) and catalase (CAT) in maize (*Zea mays* L.) under water-logged conditions were observed due to the application of benzyladenine (Liu *et al.*, 1996). Another study suggested that cytokinins such as zeatin riboside (ZR) could alleviate heat stress injury in creeping bentgrass by maintaining active antioxidants and reducing lipid peroxidation, and a high concentration (10 μmol) treatment with ZR was most effective in slowing leaf senescence and protecting cell membranes from heat injury (Liu and Huang, 2002). These studies showed that application of cytokinin results in the induction of antioxidants (Liu and Huang, 2002) and in the same vein, results from our above-mentioned studies (Chapter 1 & 3) reflected an emphasis on the involvement of antioxidants in the blackspot and blackleg-tolerant Brassica genotypes. We therefore investigated whether CKs can be helpful in delaying the development of disease caused by *A. brassicae* and *L. maculans*. Results obtained in this study revealed that CKs, specifically BAP, can reduce symptoms and delay the onset of disease caused by both pathogens, the effect being more pronounced in *L. maculans*-infected plants as compared to *A. brassicae*. We also investigated the effect of BAP and kinetin on *in vitro* fungal growth and our results suggest that BAP is able to inhibit the growth of both fungi.

Apart from plant-pathogen interactions, we were also interested in deciphering the proteomes of two isolates of *A. brassicae,* differing in virulence using 2-DE and MS techniques. This study was initiated to identify proteins potentially contributing to the observed differences in their virulence properties and functioning as putative virulence/pathogenicity factors. Several unique and quantitative differences in the

expression pattern of proteins between the two isolates of *A. brassicae* were observed. Along with hypothetical proteins with unknown functions, enzymes such as enolase, serine protease and malate dehydrogenase were observed to be present in higher abundance in the more virulent isolate as compared to the less virulent isolate. Some of these proteins including enolase and serine protease have been associated with virulence functions in diverse systems and, could be speculated to form the basis of higher virulence in the Ontario isolate of *A. brassicae*.

The overall outcome of the research presented in this dissertation suggests that an important role is played by antioxidant-mediated pathways and mechanisms involved in cell-wall reinforcement in incompatible interactions in these two pathosystems. We started this project with the aim of understanding the molecular basis of susceptibility/ resistance in different *Brassica* genotypes using 2-DE and MS based approaches. We were able to decipher the mechanisms involved in these host-pathogen interactions. We also explored alternative strategies to combat blackleg and blackspot diseases and conducted studies to test the effect of cytokinins on the onset of these diseases. While we were able to meet most of our initial objectives; this study also leads to more interesting questions that could be pursued in order to have a clearer understanding about early events that mediate the host-pathogen interactions.

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6.1. Conclusions

The availability of large-scale genomic information and improvements in the analytical methods for protein characterization have facilitated the progressive use of proteomics as an exciting tool to study different aspects of plant functions, including the plant-pathogen interaction. Plants are routinely invaded by a variety of pathogens and respond to these challenges using a combination of pre-determined defense systems and the induction of specific defense responses. Of the different phytopathogens, fungi cause enormous damage to important crops around the world. Proteomic approaches are being used to obtain a better understanding of the dynamic changes that accompany disease formation and if possible, to exploit this knowledge for the rational design of disease-tolerant crops. The purpose of the present study was to perform a comprehensive proteome-level analysis of the molecular interaction between the agronomically important *Brassica* species (and genotypes) upon infection with *L. maculans* and *A. brassicae.*

The proteomic analysis of the *A. brassicae*-Brassica pathosystem at several time-points led to the identification of several up-regulated proteins in the blackspot-tolerant genotype. These proteins are known to be involved in the production and detoxification of reactive oxygen species (ROS), ROS-mediated signaling, auxin signal transduction and other metabolic pathways. Specifically, one protein (identified as germin-like protein, GLP) was up-regulated in the blackspot-tolerant line and was present at a reduced level in the blackspot-susceptible line. Our results suggest that a drastic decrease in GLP levels in the susceptible line and its modest increase in the

tolerant counterpart may be an important factor in determining susceptibility/resistance to the pathogen. In order to test this hypothesis, the GLP cDNA was PCR amplified from *B. napus* and cloned under the control of a constitutive promoter in a plant transformation vector. This vector was mobilized into disarmed *Agrobacterium tumefaciens* and used to transform *Arabidopsis thaliana* using the floral-dip method to obtain GLP-expressing transgenics. Preliminary studies suggest that the GLP-expressing transgenics show delayed onset of disease symptoms as compared to non-transformed control plants.

We also investigated proteome-level changes in *L. maculans*-susceptible *B. napus* and -resistant *B. carinata* at different time-points following inoculation with the pathogen. A comparison of the protein profiles led to the identification of a differential abundance of enzymes involved in anti-oxidative, photosynthetic, protein processing and signaling pathways. Our results suggest that the tolerant *B. carinata* generally responds to pathogen challenge with an increase in the level of proteins with antioxidant activities whereas the susceptible species does not show such an increase. Interestingly, in the case of the susceptible *B. napus*, GLP levels declined upon pathogen challenge, an observation that was further validated by q-RT-PCR and Western bloting. Furthermore, our histological observations indicated that there is enhanced lignification around the wounding site in the tolerant *B. carinata* compared to the susceptible *B. napus*. Taken together, these results suggest that the enhanced antioxidant protein levels in *B. carinata* may be at least in part responsible for mediating tolerance to this pathogen via increased lignification in addition to detoxification of ROS.

In a parallel investigation, we studied the proteomes of two isolates of *A. brassicae* with different virulence levels. This study was initiated to identify proteins that contribute to the observed differences in their virulence properties and may function as pathogenicity/virulence factors. This study revealed quantitative differences in the expression pattern of proteins between the two isolates of *A. brassicae*. In addition to hypothetical proteins with unknown functions, enzymes such as enolase, serine protease and malate dehydrogenase were found to be present at higher levels in the virulent isolate as compared to the weakly virulent isolate. These proteins have been associated with virulence functions in diverse systems and, could be speculated to form the basis of higher virulence.

We also explored the application of cytokinin as a possible strategy for the control of these phytopathogenic fungi. Cytokinins have been known to play a role in delaying senescence in plants and, being a necrotrophic fungus, *A. brassicae* tends to infect senescing plants. We hypothesized that delayed senescence would have an effect on the onset of disease. Since, this kind of study has not been previously reported in the context of *L. maculans* and *A. brassicae*, we conducted this study. Experiments with fungal inoculations performed on both detached leaves and intact plants (pre-treated with 6-benzylaminopurine; BAP) revealed a significant reduction in disease symptoms in BAP-treated plants. Histological studies also revealed reduced mycelial growth of the pathogen in BAP-treated tissues. The *in-vitro* growth of these phytopathogens was inhibited on growth medium supplemented with BAP, suggesting that this cytokinin exerts its inhibitory effect by directly interfering with pathogen growth.

With respect to host responses, results presented in this thesis suggest that several mechanisms are involved in these host-pathogen interactions including the generation and detoxification of ROS, photosynthetic enzymes and other metabolic enzymes. Cytokinin application again suggested that by boosting the photosynthetic machinery of the plant, these diseases may be controlled to some extent or the onset of the disease may be delayed. Regarding pathogen-related studies, proteome-level differences in the two isolates of *A. brassicae*, differing in their virulence, suggest the utility of this technique in the identification of putative virulence factors and detection of virulent isolates of pathogens aiding in finding novel targets for disease management.

6.2. Future prospects

Results presented in this dissertation provide new information about the dynamic changes in the *B. napus* and *B. carinata* proteome upon inoculation with two phytopathogenic fungi, namely *A. brassicae* and *L. maculans*. Several enzymes involved in the antioxidant pathways were found to be differentially expressed in the tolerant *Brassica* genotypes following pathogen challenge. Preliminary results obtained with transgenic *Arabidopsis thaliana* constitutively over-expressing one such enzyme, namely germin-like protein (GLP), suggest that the transgenics lines were more tolerant to pathogen challenge than their non-transformed counterparts. The systematic exploration of these two pathosystems establishes the utility of using proteomic-based approaches in illuminating some aspects of the mechanisms involved in the interactions between *Brassica* species and *L. maculans* and *A. brassicae*. Furthermore, the proteome-level differences between two *A. brassicae* isolates differing in virulence could serve as biomarkers to detect new and potentially pathogenic isolates. The inhibitory role of one of the cytokinins (i.e. BAP), to inhibit the in planta and in vitro growth of these phytopathogens was also investigated. The current research endeavor, which aims to generate durable resistance against these diseases, is a preliminary step in this direction as many aspects remain to be addressed in order to achieve the ultimate goal.

Some of the future research directions to pursue may be as follows:

A. Chapters 2 and 4

1) Results described in these studies show that several enzymes in the antioxidant pathway are up-regulated in tolerant *Brassica* species upon pathogen inoculation and this could be a function of the plants ability to resist the damage caused by the pathogen. Transgenic *A. thaliana* over-expressing GLP showed fewer disease symptoms and appeared more tolerant to the pathogen challenge, compared to non-transformed plants. These results are preliminary and follow-up experiments need to be performed to confirm these results.

    a) It would be interesting to test some of the other gene candidates such as type II peroxiredoxin and 2-cys peroxiredoxin (corresponding to other up-regulated enzymes) in *A. thaliana* so as to evaluate their role(s) in disease tolerance.

    b) Pyramiding of genes that give a positive response with regard to disease tolerance could also be assessed in *A. thaliana* to enhance the plants ability to withstand pathogen-induced stress.

    c) If these genes show the expected behavior in the model plant, *A. thaliana*, then eventually the same strategy could be attempted in *B. napus* to check the utility of this approach in the amelioration of disease.

2) A specific interaction between the avirulence gene product in the pathogen and the corresponding R-gene product induces the hypersensitive response and activates downstream defense responses. In addition to the R-gene based

defense, plants rely on the recognition of general elicitors, called pathogen-associated molecular patterns, to identify potential pathogens and activate basal defense mechanisms that include generation of reactive oxygen species, biosynthesis of ethylene, activation of MAPK cascade and the downstream activation of defense gene expression.

Very little is known about early events in signal transduction cascades activated by R-gene or general elicitors, specifically those involving post-translational modifications via protein phosphorylation-dephosphorylation. Although protein kinases and phosphatases have been associated with defense signaling and resistance, their exact role and their substrates have remained elusive in plants. The large scale identification of phosphorylation sites as well as the kinetics and stoichiometry of the phosphorylation status may enable us to characterize early signal transduction cascades and defense responses. However, such changes are very difficult to detect using a standard 2-DE approach that relies on major changes in protein abundances. The development of powerful techniques can greatly enhance the sensitivity and reliability of information obtained with conventional 2-DE approaches. One such technique, namely 2-DE difference gel electrophoresis (DIGE), involves pre-incubating protein samples with activated fluorescent dyes to label lysine (or cysteine) residues with a sensitive tag that can be used to quantify the abundance of that protein in solution and is especially suitable for monitoring processes in which few changes in protein expression or posttranslational modification are expected (Benschop *et al.*, 2007). The study of proteome-

level changes in *Brassica* upon inoculation with the aforementioned fungal pathogens at earlier time-points using DIGE-based 2-DE may provide valuable insights into early events following host-pathogen interactions.

B. Chapter 3

This chapter describes the proteome-level variation between two differentially virulent isolates of *Alternaria brassicae*. This study resulted in the identification of several unique/hypothetical proteins with unknown functions and increased relative abundance of some proteins (such as enolase and serine protease) in the more virulent Ontario isolate. It is likely that some of these proteins such as enolase and serine protease may play roles in determining virulence and provides an exciting opportunity to probe the functions of these proteins using the targeted gene disruption approach in the *A. brassicae* background. Techniques for genetic transformation and targeted gene disruption that have been established for the closely related species, *Alternaria brassicicola,* could be adapted for experiments with *A. brassicae*. In a recent report, Kim *et al.* (2007) have successfully adopted this strategy to establish the role of a non-ribosomal peptide synthetase gene in conidial wall construction of *A. brassicicola.*

In addition to the proposed gene disruption experiment, another technique that could be used to study the putative role(s) for the identified proteins involves the use of recombinant antibody technology. Generating transgenic *A. brassicae* expressing antibody fragments such as single chain, variable fragments (scFv) intracellularly could provide valuable information regarding the roles for

some of the hypothetical proteins identified in our study comparing the differentially virulent *A. brassicae* isolates. Immunomodulation of selected proteins could result in phenotypic changes affecting the virulence or pathogenicity of the fungus thereby demonstrating their importance for inducing disease in susceptible plants. These intracellularly expressed scFv antibodies (perhaps labeled with green fluorescent protein) could also be useful in determining the localization within the fungus of many of the identified proteins, which could provide important clues as to their functions.

Generating transgenic plants expressing scFv antibodies that specifically recognize and potentially inhibit selected proteins may also provide some information regarding whether the proteins are virulence or pathogenicity factors. An inhibition of the activity of a protein exposed on the cell surface that is unique to, or more abundant in, the more virulent *A. brassicae* resulting in a decrease in virulence may provide evidence as to the importance of the identified protein for pathogenicity. Furthermore, the application of recombinant antibody technology to confer increased tolerance to fungal disease in plants has previously been demonstrated in *A. thaliana* and a similar strategy could be applied here (Peschen *et al.*, 2004).

C. Chapter 5

In this chapter, we showed that application of one of the cytokinins, BAP, effectively inhibits both the *in planta* and *in vitro* growth of both *L. maculans* and

A. brassicae. Additional experiments that could be undertaken to further investigate this phenomenon are as follows:

a) Proteome-level investigation of BAP-treated and non-treated plants with and without infection. This may provide a clearer idea about the events unfolding at the molecular level in the plant and the pathogen, some of which might be crucial in explaining the observed tolerance of BAP-treated tissues to pathogen infection and the inhibition of pathogen growth on BAP-supplemented media.

b) Measurements of endogenous levels of BAP in the plant tissues both before and after pathogen inoculation. Since the signaling and metabolic pathways of several phytohormones (such as auxins, ethylene and abscisic acid) operate in a synergistic or antagonistic manner, it would also be useful to estimate the levels of these hormones.

c) To test the disease tolerance ability of cytokinin-overexpression and -knockout mutants.

Levels of anti-oxidant enzymes such as superoxide dismutase and peroxidase are known to be induced upon cytokinin application (Liu *et al.*, 1996; Liu and Huang, 2002). The presence of this pathway could be easily tested via enzyme assays that may validate or rule-out the role of this pathway in cytokinin-mediated amelioration of pathogenesis.

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Appendix 1

A.1. Constitutive over-expression of GLP cDNA of *B. napus* in *Arabidopsis thaliana*

Our proteomics-based results suggested that the dramatic decrease in the spot intensity of GLP in the *A. brassicae*-susceptible line and its modest increase in the *A. brassicae*-tolerant counterpart could be an important factor in determining susceptibility/resistance to the pathogen. Furthermore, gene-expression analysis of GLP using q-RT-PCR revealed increase in GLP transcript at 24 and 48 h in *A. brassicae*-tolerant line following infection whereas decrease in *A. brassicae*-susceptible line at the same time-points. Therefore, the decrease in GLP transcript and protein levels at 24 h in the susceptible line after pathogen challenge may be important for compatible host-pathogen interactions. In order to test this hypothesis, the *B. napus* GLP cDNA was cloned using RT-PCR and introduced into the plant transformation vector (pKYLX71) which controls the expression of the introduced cDNA by the constitutive CaMV 35S promoter. This vector was mobilized into disarmed *Agrobacterium tumefaciens* (GV3101) and used to transform *Arabidopsis thaliana* using the floral-dip method to obtain GLP transgenics. Preliminary studies suggest that the homozygous GLP transgenics displayed delayed disease symptoms as compared to non-transformed control plants especially lines GLP-2, 19 and 20 (See Fig. A-1). These experiments are currently being repeated to verify these results.

Figure A-1. Effect of *A. brassicae* (UAMH7476) infection on different *A. thaliana* genotypes. GLP stands for different homozygous lines of germin-like protein transgenics.